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Further Studies on the Relationship between the Genotype and the Mammary Tumor Agent in Mice [1]

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Genetic control over the propagation and transmission of the mammary tumor agent was clearly shown in a study carried out in this laboratory, the results of which were published in 1945 (*1*). C3HB × C3H backcross females and C3HB × B backcross females that had comparable (C3H × B)F_1 mothers and therefore had received comparable mammary tumor agent and that were also the same with respect to other possible inherited maternal influences differed not only in their incidences of mammary gland tumors but also in their ability to transmit the agent, as indicated by a difference in the occurrence of tumors in their (B × C3H)F_1 foster-nursed test females. The only explanation for this difference between the two groups of backcross females was that it was due to genotypic differences resulting from the C3HB × C3H backcross females having had genetically susceptible strain C3H fathers, whereas the C3HB × B backcross females had resistant strain C57BL fathers.

The demonstration of this gene-agent relationship and the similarity of this situation to other gene-cytoplasmic factor relationships, such as the gene-*kappa* relationship in paramecia (*2*), provoked many questions regarding the nature of the mammary tumor agent and the control exercised by genetic factors over its survival and propagation. How strong is this gene-agent bond and how readily can the agent be eliminated by changing the genotype? Andervont (*3*) found that strain C57BL mice that were given the agent could transmit it through their milk to susceptible foster-nursed females, which in turn could transmit it, but their own C57BL female offspring could not transmit the agent. Earlier Murray and Little (*4*) had found that, after starting with strain DBA females with the agent and outcrossing them to C57BL males and then backcrossing the female offspring to C57BL males, the agent was no longer present in the females of the eighth backcross generation. This was indicated by the fact that the females of that generation did not develop tumors, nor did their genetically susceptible offspring that resulted from outcrossing to strain DBA males. Questions remained as to how soon the agent dis-

[1] Received for publication January 5, 1956.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

appeared under such circumstances and whether it actually was eliminated or merely became "masked," and, if so, whether it could be reactivated by again creating a proper host genotype.

There were also questions regarding the origin of the agent. Was it extrinsic or intrinsic? Bittner (*5*) reported the appearance of the agent *de novo* in an Ax stock that had presumably been freed of the agent.[3] Could the agent possibly arise as a mutation of some normal cell constituent? Woods and duBuy (*6*) reported evidence suggesting that phytopathogenic viruses might arise from mitochondria and their derivatives. [Arguments against their hypothesis have since been presented (*7*).] It seemed reasonable that if the mammary tumor agent had arisen from such intrinsic origin it might be caused to arise in a stock by building up a genetic background in that stock making it susceptible to the agent.

In 1946, an extensive breeding and foster-nursing project was set up to shed light on these and other related questions. This was a long-term project that required the ensuing 8 years for completion. Observations were made on the occurrence of tumors in 4,049 female mice of various specific genotypes. The results have clarified some concepts regarding the origin and nature of the mammary tumor agent and have yielded additional information on the genic control of the agent and on its transmission.

During this 8-year period observations from similar types of experiments have been made and reported by others. Thus, some of the results reported herein are not new. It seemed advisable, however, not to publish until the project was completed so that a comprehensive report of the whole study could be made, thus allowing the significance of different factors to be more accurately estimated.

Materials and Methods

The three strains of mice used in this study were strain C3H, strain C3Hf, and strain C57BL. Strain C3H has the agent and is genetically susceptible to it. The females have a mammary tumor incidence of approximately 95 to 100 percent, with an average tumor age of approximately 8 months in the breeders and approximately 11 months in the virgins. Strain C3Hf was derived from a litter of strain C3H mice taken from the mother by cesarean section and foster-nursed upon a strain C57BL female. Thus, strain C3Hf does not have the mammary tumor agent, although genetically susceptible to it. When bred intensively, strain C3Hf females have a mammary tumor incidence of 38 percent at an average age of 20 months. In virgin females the incidence is less than 5 percent. Strain C57BL does not have the agent and is genetically resistant to it. No mammary tumors have occurred since the strain was received from Dr. H. B. Andervont in 1945. All three of these strains have been extensively used in this laboratory in work previously reported (*1,8,9*).

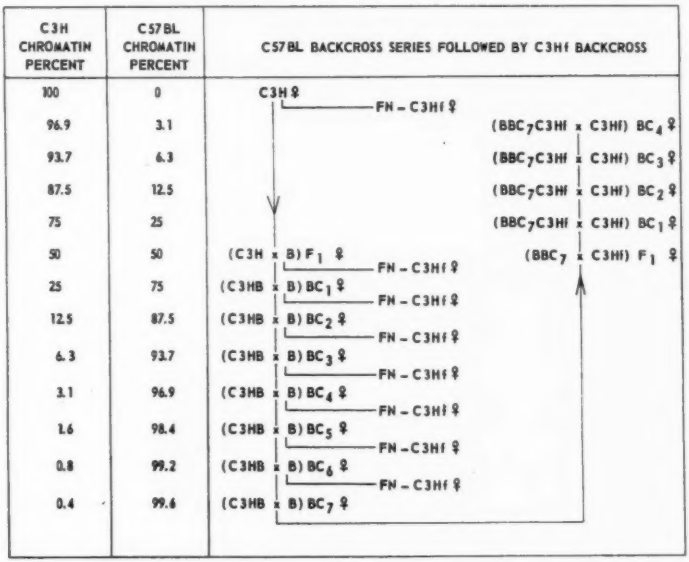
In order to study the genetic control over the propagation and transmission of the agent, a C57BL backcross series was bred, and this was

[3] Dr. L. Dmochowski has mentioned in a published discussion a similar observation in strain C57BL mice, J. Nat. Cancer Inst. 15: 785-787, 1954.

followed by a C3Hf backcross series as outlined in text-figure 1. Strain C3H females were outcrossed to strain C57BL males. The resultant (C3H × B)F_1 females were then backcrossed to C57BL males, as were females of the succeeding backcross generations. By this system of matings, resistant strain C57BL chromatin was introduced and progressively increased in succeeding generations at a theoretical rate of increase indicated in the text-figure. The backcrossing was continued to the seventh generation, in which the theoretical average proportion of C57BL chromatin was 99.6 percent. Since a continuous line of females descended from the original C3H mothers was maintained, it was possible to observe the survival of the agent against the various concentrations of resistant chromatin in the successive generations.

In order to ascertain whether the agent would be eliminated by the introduction of C57BL chromatin, and, if so, how readily it would be eliminated, it was planned at the outset to mate 20 females in each generation and to rear 5 female offspring from each of these matings. Thus, each generation would have 100 females. Twenty of these would be used as breeders and 80 kept as virgins. The original group of C3H females, of course, consisted of only the 20 breeders. Some generations included a few more than 20 breeding females and others had a few less breeders and virgins than originally planned, but each generation had an adequate number to establish a tumor incidence.

Since the females of each backcross generation would be genetically variable and would be expected to be resistant to the mammary tumors



TEXT-FIGURE 1.—Outline of the C57BL backcross series followed by the C3Hf backcross series. The theoretical average percentage of C3H chromatin and of C57BL chromatin is indicated for each generation.

in varying degrees, it was planned that each of the breeding females was to foster-nurse five C3Hf test females. These C3Hf females, while not carrying the mammary tumor agent, would yet be uniformly highly susceptible to it and would furnish a satisfactory test for the occurrence of the agent in each generation. A total of 100 C3Hf foster-nursed test females was planned for each generation. This number was not always attained but in each generation the number was high enough to disclose the presence or absence of the agent.

By the time the seventh C57BL backcross generation was produced it had become obvious that the mammary tumor agent, as judged by the production of tumors, had disappeared several generations earlier. However, there was still the question of whether this failure to produce tumors represented complete elimination of the agent or merely a latent state of the agent. It was reasoned that if it had become latent because of the concentration of resistant strain C57BL chromatin, surely it could be reactivated by again building up a susceptible strain C3H chromatin background for it. Accordingly, 20 of these seventh backcross females were outcrossed to C3Hf males (C3Hf males being used to insure against the possibility of introducing the agent by the male), and the resultant ($BBC_7 \times$ C3Hf)F_1 females were backcrossed to C3Hf males, as were the females of the succeeding generations, until the fourth backcross generation. Again it was planned to produce in each generation 100 females, 20 to be used as breeders and 80 to be kept as virgins, with the exception of the fourth backcross in which all the females were to be kept as virgins. Since these females would be highly susceptible to the agent, 50 percent

C57BL CHROMATIN PERCENT	C3H CHROMATIN PERCENT	C3H BACKCROSS SERIES
100	0	C57BL ♀
		FN – C3Hf ♀
50	50	(B x C3H) F_1 ♀
		FN – C3Hf ♀
25	75	(BC3H x C3H) BC_1 ♀
		FN – C3Hf ♀
12.5	87.5	(BC3H x C3H) BC_2 ♀
		FN – C3Hf ♀
6.3	93.7	(BC3H x C3H) BC_3 ♀
		FN – C3Hf ♀
3.1	96.9	(BC3H x C3H) BC_4 ♀
		FN – C3Hf ♀
1.6	98.4	(BC3H x C3H) BC_5 ♀
		FN – C3Hf ♀
0.8	99.2	(BC3H x C3H) BC_6 ♀
		FN – C3Hf ♀
0.4	99.6	(BC3H x C3H) BC_7 ♀

TEXT-FIGURE 2.—Outline of the C3H backcross series. The theoretical average percentage of C57BL chromatin and of C3H chromatin is indicated for each generation.

of the chromatin of the F_1 generation being C3H and, theoretically, an average of 96.9 percent of that of the fourth backcross generation, it was considered that these females would supply adequate tests for the agent and thus there were no foster-nursed C3Hf test females for these generations.

To gain some facts bearing on the speculation as to the possible intrinsic origin of the mammary tumor agent, the reciprocal of the above system of matings was outlined and carried out (text-fig. 2). In this series, later referred to as the 3CH backcross series, C57BL females without the agent were outcrossed to C3H males, and the female offspring were backcrossed to C3H males through seven backcross generations. If the agent could arise intrinsically it might be expected to appear as the susceptible C3H

C57BL CHROMATIN PERCENT	C3H CHROMATIN PERCENT	C3Hf BACKCROSS SERIES
100	0	C57BL ♀
50	50	(B x C3Hf) F_1 ♀
25	75	(BC3Hf x C3Hf) BC_1 ♀
12.5	87.5	(BC3Hf x C3Hf) BC_2 ♀
6.3	93.7	(BC3Hf x C3Hf) BC_3 ♀
3.1	96.9	(BC3Hf x C3Hf) BC_4 ♀
1.6	98.4	(BC3Hf x C3Hf) BC_5 ♀
0.8	99.2	(BC3Hf x C3Hf) BC_6 ♀
0.4	99.6	(BC3Hf x C3Hf) BC_7 ♀

FN – C3Hf ♀

TEXT-FIGURE 3.—Outline of the C3Hf backcross series. The theoretical average percentage of C57BL chromatin and of C3H chromatin is indicated for each generation.

chromatin background was provided. Since the possibility existed that the C3H males themselves might transmit the agent, a second series was outlined in which C3Hf males without the agent were used (text-fig. 3). This series is referred to as the C3Hf backcross series. Appearance of the agent in both series would indicate intrinsic origin of the agent, whereas evidence of the agent in the C3H backcross series and not in the C3Hf backcross series would indicate transmission of the agent by the C3H males.

In both the C3H and the C3Hf backcross series it was planned to utilize a number of animals similar to that in the first series, *i.e.*, to start with 20 C57BL breeding females in each series, which would produce 100 females for the next generation—20 to be used as breeders and 80 kept as

virgins. The 20 breeders of this generation would in turn produce 100 females for the next generation, and so on. Both of these series also were extended to the seventh backcross generation, at which time the theoretical average proportion of C3H chromatin was 99.6 percent. Again it was planned that in addition to having each of the 20 breeding females rear 5 of her own female offspring she would foster-nurse 5 C3Hf test females, thus supplying 100 C3Hf test females for each but the last generation of each series. As in the first series, the full number was not obtained for each generation, but each generation had an ample number for determining whether the agent was present and for establishing a tumor incidence.

At weaning, the females to be kept as virgins in all series were set aside in cages, eight females to a cage, as were the breeding females after they had reared their five female offspring and foster-nursed their five C3Hf test females. The animals were housed in wooden cages from 1946 to 1949, after which the colony was housed in plastic cages. Throughout the study the mice were given an unlimited supply of Derwood pelleted food and tap water.

The mice were kept under observation for tumor development throughout their life span. All animals were necropsied when they developed large tumors or were found to be moribund. A few were found dead and were necropsied if there was not too much *post-mortem* change. The tumors were fixed routinely in Fekete's modification of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic diagnosis.

Occurrence of Mammary Gland Tumors

C57BL backcross series and subsequent C3Hf backcross series.—The incidence of mammary gland tumors in breeding and virgin females in each generation of the C57BL backcross series and in the foster-nursed C3Hf test females for each generation are presented in table 1, along with the incidence in breeding and virgin females of the subsequent C3Hf backcross series. The incidence of 90 percent, with an average tumor age of 9 months in the original 20 C3H females, was in accordance with what has been observed in this strain. The incidence of 94 percent, with an average tumor age of 10 months in the foster-nursed C3Hf females, was what could have been expected in virgin C3H females with the agent. As has been observed before, the genetic difference between the C3H females and the (C3H $\times$ B)F_1 females was manifested more significantly as an increased average tumor age in the F_1 females than as an incidence difference, although in both the breeders and virgins the incidence was slightly lower in the F_1 females. This difference is noted especially in a comparison of the virgin F_1 females, which had an incidence of 85 percent and an average tumor age of 15 months, with the C3Hf test females, that were foster-nursed by the C3H mothers and that had a tumor incidence of 94 percent at an average tumor age of 10 months. Since both groups

TABLE 1.—*Mammary tumors in the C57BL backcross series (C3H ♀ ♀ outcrossed to C57BL ♂ ♂ followed by backcrosses to C57BL ♂ ♂) and in the subsequent C3Hf backcross series (BBC_7 ♀ ♀ outcrossed to C3Hf ♂ ♂ followed by backcrosses to C3Hf ♂ ♂)*

Generation	Breeders					Virgins					Foster-nursed virgins				
	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)
		No.	%				No.	%				No.	%		
C3H	20	18	90	9	21										
FN* – C3Hf	—	—	—	—	—	—	—	—	—	—	88	83	94	10	26
(C3H × B†)F_1	19	14	74	11	27	71	60	85	15	22					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	80	55	69	11	24
(C3HB × B)BC_1	17	4	24	20	18	65	6	9	17	22					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	69	54	78	12	24
(C3HB × B)BC_2	19	1	5	21	22	54	0	0	—	24					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	97	18	19	13	22
(C3HB × B)BC_3	19	0	0	—	21	77	1	1	26	23					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	93	1	1	21	20
(C3HB × B)BC_4	20	0	0	—	22	64	0	0	—	21					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	89	0	0	—	16
(C3HB × B)BC_5	17	0	0	—	21	70	0	0	—	22					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	76	3	4	24	23
(C3HB × B)BC_6	23	0	0	—	20	37	1	3	22	25					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	89	4	4	27	24
(C3HB × B)BC_7	33	0	0	—	25	45	0	0	—	23					
(BBC_7 × C3Hf)F_1	21	2	10	31	27	79	6	8	25	28					
(BBC_7C3Hf × C3Hf)BC_1	19	1	5	25	22	81	6	7	25	25					
(BBC_7C3Hf × C3Hf)BC_2	18	2	11	21	21	78	1	1	24	24					
(BBC_7C3Hf × C3Hf)BC_3	20	2	10	21	19	80	4	5	20	22					
(BBC_7C3Hf × C3Hf)BC_4	—	—	—	—	—	103	3	3	21	22					

*FN = Foster-nursed. †B = C57BL.

Vol. 16, No. 6, June 1956
379436—56——2

received comparable milk agent the difference must be considered a genetic difference.

A marked reduction in incidence and increase in tumor age was noted in the first backcross generation, especially in the virgins where the incidence decreased from 85 percent in the F_1 to 9 percent in the first backcross. That the F_1 mothers had transmitted rather ample amounts of the agent to these first backcross females was indicated by the relatively high incidence of tumors in the C3Hf test females that were foster-nursed by the F_1 mothers. Thus, this marked reduction in incidence of tumors in the first backcross females was largely an expression of reduction of C3H chromatin, which in turn reduced the propagation of the agent in these females and also their response to the agent. It should be noted, however, that the F_1 females did not transmit the agent as well as their C3H mothers had, as noted in the comparison of the incidence of tumors in the C3Hf test females foster-nursed by the F_1 females with the incidence in those foster-nursed by the C3H females.

By the second backcross generation it appeared that very little mammary tumor agent remained. The females of this generation had surely received the agent from their first backcross mothers, for 78 percent of the C3Hf test females that were foster-nursed by these first backcross females developed tumors. Nevertheless very little of the agent was propagated in the second backcross females, for none of the virgin females and only one of the breeders developed a tumor. Of the C3Hf females that foster-nursed these second backcross females, 19 percent developed tumors at a rather early age indicating that some of the second backcross females were transmitting the agent. In checking the pedigree chart it was evident that transmission of the agent was limited to three second backcross females, two of which did not develop tumors although they lived to 24 and 22 months of age. The third was the only second backcross female that developed a tumor. Of the 5 C3Hf females foster-nursed by one of these nontumorous backcross females, 4 developed tumors at an average age of 12 months, and of the 5 C3Hf test females foster-nursed by the other, all developed tumors at an average age of 12 months also. Of the 5 C3Hf test females foster-nursed by the second backcross female that later developed a tumor, 3 developed tumors at an average age of 13 months. The other tumors occurring in this group of C3Hf test females were scattered throughout the other litters and occurred at too advanced an age to indicate the presence of the agent.

By the third backcross generation, however, it was certain that the agent had been eliminated or was no longer active. None of the breeders of the third backcross had a mammary tumor and only one of the virgins had a tumor, and it appeared when the female was 26 months old. Furthermore, there was no evidence that any of the breeding females of this generation transmitted the agent, for only 1 of their 93 C3Hf test females had a tumor, and it appeared when the female was 21 months old.

As noted in table 1, only one tumor arose in the subsequent generations of this series. A few occurred in the C3Hf test females that were foster-

nursed by females of these subsequent generations, but the females were so old when the tumors appeared that the tumors could not be considered as evidence of the agent.

Thus it was shown that in this system of backcrossing to the resistant strain C57BL males the agent had survived in an active state in only a few of the females of the second backcross generation and in none of those of the third backcross generation. Such prompt disappearance of the agent would indicate that relatively few genes are involved in the control of its propagation and transmission. Another possible, although less likely, explanation is that there may be many genes involved, but approximately the full complement is necessary for survival of the agent. Had there been many genes, with an additive effect, one would have expected the agent to have persisted in reduced amounts through more generations.

The question that arose at this point was whether the agent had been completely eliminated through the alteration of genotype of the host or had merely been caused to become latent or inactive. Were the latter true one could expect the agent to be activated by restoring a C3H genetic background. Such a background was restored by mating these seventh-backcross-generation females to C3Hf males and backcrossing the female offspring to C3Hf males through four backcross generations. Fifty percent of the chromatin of these F_1 females would be C3H chromatin, and by the fourth backcross generation the theoretical average percentage of C3H chromatin would be 96.9 percent. As noted in table 1, a few mammary gland tumors occurred in these F_1 females and also in the females of each of the four backcross generations, but there were no more than could have been expected in C3Hf females without the agent, and all the tumors occurred when the females were of advanced ages. Furthermore, there was no increase in tumor incidence through succeeding generations. Thus, in the 499 females of these 5 generations there was no evidence of reactivation of a latent agent. It could, therefore, be concluded that in the C57BL backcross series, through alteration of the genotype of the hosts, the agent had been completely eliminated.

C3H backcross series.—The mammary gland tumors occurring in the C3H backcross series are recorded in table 2. There was no indication that the agent could have occurred in the C57BL females with which the series was started, for no tumors occurred in any of the original 17 C57BL females that were mated to C3H males and none occurred in any of the 81 C3Hf test females that were foster-nursed by the 17 C57BL females. This observation is in line with our belief that the agent does not occur in our line of strain C57BL.

Tumors did occur, however, in some of the females of the F_1 and subsequent backcross generations, the incidence, in general, increasing in each successive generation. In the fifth and later backcross generations the incidence was relatively high and the average tumor age comparatively low, indicating that the agent must be present. Similar evidence for the presence of the agent also appeared in the groups of C3Hf test females that were foster-nursed by the backcross females of these generations.

TABLE 2.—*Mammary tumors in the C3H backcross series (C57BL ♀ ♀ outcrossed to C3H ♂ ♂ followed by backcrosses to C3H ♂ ♂)*

Generation	Breeders					Virgins					Foster-nursed virgins				
	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)
		No.	%				No.	%				No.	%		
C57BL	17	0	0	—	22										
FN* – C3Hf	—	—	—	—	—	—	—	—	—	—	81	0	—	—	22
(B† × C3H)F_1	17	1	6	20	25	68	0	0	—	28					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	90	5	6	13	23
(BC3H × C3H)BC_1	19	1	5	17	23	67	4	6	14	25					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	64	6	9	16	22
(BC3H × C3H)BC_2	17	0	0	—	22	70	1	1	26	23					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	59	4	7	15	21
(BC3H × C3H)BC_3	20	7	35	15	20	36	1	3	19	20					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	67	15	22	12	16
(BC3H × C3H)BC_4	16	7	44	11	14	48	9	19	12	18					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	41	19	46	11	20
(BC3H × C3H)BC_5	11	9	82	11	19	27	12	44	15	16					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	33	22	67	13	14
(BC3H × C3H)BC_6	20	14	70	9	13	14	8	57	12	20					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	67	43	64	13	20
(BC3H × C3H)BC_7	23	18	78	9	9	68	42	62	13	19					

*FN = Foster-nursed.
†B = C57BL.

The entrance of the agent into this series was much more striking in the pedigree chart where one could pick out the various females into which the agent had been introduced. In the F_1 generation 1 female that had a tumor at 20 months had definitely received the agent, presumably from the male with which she was mated, by the time she nursed her second litter. She reared none of her first litter but did rear one C3Hf female that she foster-nursed at the time of her first litter, and this test female died without a tumor at 23 months of age. She reared 5 of her second litter, of which 3 had mammary tumors at 5, 8, and 12 months of age and 2 died without mammary tumors at 21 and 30 months of age. The occurrence of these tumors indicated that this F_1 female by this time had the agent. Three C3Hf test females, foster-nursed by the F_1 female after her third litter was born, confirmed the presence of the agent for they developed mammary tumors at 6, 9, and 9 months of age. Thus, it was readily apparent that the F_1 female had received the agent from the male with which she was mated, and even though the amount of the agent she received may have been very low it was built up to produce a high incidence of tumors in her own and her foster-nursed offspring at a relatively low average tumor age.

Similarly, one of the first backcross females became infected with the mammary tumor agent from the male during her breeding period. Her first litter was discarded at weaning time but of her second litter five females were reared. These all died without mammary tumors at 21 to 24 months of age. However, of 4 C3Hf test females foster-nursed by this first backcross female after the birth of her third litter, all 4 developed mammary gland tumors at 10 to 16 months of age. This indicated that by the time of her third litter the first backcross female had the agent in such quantity as to give an unquestionable positive test in the females that she foster-nursed. The five second backcross females of this second litter, none of which developed a tumor, were used as breeders for producing third backcross females. There was no evidence of the agent in the descendants of four of these second backcross females but in descendants of the fifth female there was strong evidence of the agent. No young of her first litter were reared and only one female of her second litter, and this female apparently did not receive the agent, for none of the 4 C3Hf test females that she foster-nursed developed a tumor. This second backcross female foster-nursed two C3Hf test females after her third litter, and both of these females developed tumors at 10 and 11 months of age, indicating that by her third litter this second backcross female had the agent and was able to transmit it. She reared 4 females of her 4th litter, all of which developed mammary tumors at 7 to 12 months of age. At this time she also foster-nursed a C3Hf test female, which developed a mammary tumor at 12 months of age. These four third backcross females, all of which had tumors, transmitted the agent to their descendants through the subsequent backcross generations. Seventy-one of the 91 breeding and virgin females of the 7th backcross generation were descendants of these 4 females, and of these 71 descend-

ants 59, or 83 percent, developed tumors at an average age of 12.1 months, an incidence and average tumor age comparable with strain C3H virgin females.

The occurrence of tumors in descendants of other females in this whole C3H backcross series was similar to that in the C3Hf line and in the C3Hf backcross series. The tumors occurred at advanced ages in females scattered throughout the pedigree chart. Thus, it appeared that occasionally a female would become infected with the agent from the male with which she was mated and, although the amount of the agent she received may have been low, it was built up to such concentration as to give an unquestionably positive test in her own offspring and in the C3Hf test females that she foster-nursed.

C3Hf backcross series.—The occurrence of mammary tumors in the C3Hf backcross series is shown in table 3. As in the C3H backcross series, there was no evidence of the agent in the 20 C57BL females with which the series was started. None of these 20 females had tumors and, although a few tumors occurred in their foster-nursed C3Hf test females, the incidence was not significantly higher than has been observed in non-fostered C3Hf females. Furthermore, the tumors arose at such an advanced age—average of 25 months—that they could not be considered as evidence of the presence of the agent. In the F_1 generation and in each of the subsequent backcross generations a few mammary tumors appeared, as they did in the C3Hf test females, for all but one generation, but in no group was the incidence high enough nor the average tumor age low enough to indicate the presence of the agent. The incidence of tumors tended to increase slightly in the breeding females with an increase in proportion of C3H chromatin, but it did not reach the incidence of 38 percent observed for C3Hf females bred extensively (*8*). No increase in tumor incidence was evident in the virgin backcross females of successive generations nor in the test C3Hf females of the successive generations, whereas an increase would have been expected had the agent been introduced. Furthermore, the females with tumors were scattered throughout the pedigree chart rather than tending to be limited to certain lines, as in the C3H backcross series into which the agent was introduced.

Parent-Offspring Correlation

In order to gain further information on the presence or absence of the mammary tumor agent in the various groups, females of the various series were tabulated with respect to whether they developed a mammary tumor and whether their mother or foster mother developed a tumor. Were the agent present in some of the females, one would expect a positive parent-offspring correlation. The results of this tabulation supported the former conclusions as to which groups were infected with the agent.

In the C57BL backcross series (table 4) a positive correlation appeared down to the third backcross generation, indicating the presence of the agent in at least some of the females throughout these generations.

TABLE 3.—*Mammary tumors in C3Hf backcross series (C57BL ♀♀ outcrossed to C3Hf ♂♂ followed by backcrosses to C3Hf ♂♂)*

Generation	Breeders					Virgins					Foster-nursed virgins				
	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)	Number	With tumor		Avg. tumor age (*mos.*)	Avg. age without tumor (*mos.*)
		No.	%				No.	%				No.	%		
C57BL	20	0	0	—	23.7										
FN* – C3Hf	—	—	—	—	—	—	—	—	—	—	90	6	7	25	24
(B† × C3Hf)F_1	24	0	0	—	27	74	2	3	25	28					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	77	1	1	19	22
(BC3Hf × C3Hf)BC_1	19	2	11	22	23	68	1	1	22	25					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	79	2	3	22	23
(BC3Hf × C3Hf)BC_2	16	1	6	21	22	56	1	2	21	22					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	61	1	2	19	19
(BC3Hf × C3Hf)BC_3	23	5	22	20	21	29	1	3	24	21					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	78	1	1	18	19
(BC3Hf × C3Hf)BC_4	19	2	11	22	13	76	2	3	24	18					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	63	3	5	19	17
(BC3Hf × C3Hf)BC_5	13	4	31	20	18	46	1	2	24	19					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	39	0	0	—	20
(BC3Hf × C3Hf)BC_6	19	1	5	23	17	21	2	10	25	21					
FN – C3Hf	—	—	—	—	—	—	—	—	—	—	57	1	2	15	20
(BC3Hf × C3Hf)BC_7	23	3	12	22	15	49	1	2	21	21					

*FN = Foster-nursed.
†B = C57BL.

TABLE 4.—*Parent-offspring correlation in C57BL backcross series and absence of parent-offspring correlation in subsequent C3Hf backcross series*

Generation	Offspring of mothers with tumors						Offspring of mothers without tumors					
	Breeders		Virgins		Foster-nursed virgins		Breeders		Virgins		Foster-nursed virgins	
	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors
C3H	20	18	0	0	—	—	0	0	0	0		
FN* – C3Hf	—	—	—	—	82	80	—	—	—	—	6	3
(C3H × B†)F_1	13	9	70	60	—	—	6	5	1	0		
FN – C3Hf	—	—	—	—	52	50	—	—	—	—	28	5
(C3H × B)BC_1	17	4	44	6	—	—	0	0	21	0		
FN – C3Hf	—	—	—	—	16	16	—	—	—	—	53	38
(C3H × B)BC_2	5	0	8	0	—	—	14	1	46	0		
FN – C3Hf	—	—	—	—	5	3	—	—	—	—	92	15
(C3H × B)BC_3	0	0	5	0	—	—	19	0	72	1		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	93	1
(C3H × B)BC_4	0	0	0	0	—	—	20	0	64	0		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	89	0
(C3H × B)BC_5	0	0	0	0	—	—	17	0	70	0		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	76	3
(C3H × B)BC_6	0	0	0	0	—	—	23	0	37	1		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	89	4
(C3H × B)BC_7	0	0	0	0			33	0	45	0		
(BBC_7 × C3Hf)F_1	0	0	0	0			21	2	79	6		
(BBC_7C3Hf × C3Hf)BC_1	0	0	10	0			19	1	71	6		
(BBC_7C3Hf × C3Hf)BC_2	0	0	4	0			18	2	74	1		
(BBC_7C3Hf × C3Hf)BC_3	2	1	5	1			18	1	75	3		
(BBC_7C3Hf × C3Hf)BC_4	0	0	10	0			0	0	93	3		

*FN = Foster-nursed. †B = C57BL.

Considering that the agent was under the influence of the genotype, one concludes that this positive correlation resulted indirectly from the genetic variation that would occur in these generations. In the third and subsequent backcross generations the few tumors that occurred were found in offspring of females that did not develop tumors. However, only five females in these generations were offspring of females that developed tumors.

In the subsequent C3Hf backcross series there was no evidence of a positive parent-offspring correlation, although there was some increase in incidence of tumors because of the increase in genetic susceptibility. There was, therefore, no evidence that the tumors in this series were the result of the mammary tumor agent. Furthermore, no genetic variation was detected in this manner in the F_1 and backcross generations. The absence of such variation here would suggest that at least some of the principal genes involved in the development of tumors in the absence of the agent were dominant.

In the C3H backcross series (table 5) a positive parent-offspring correlation was observed in most of the groups beyond the F_1 generation. Such correlation supports the conclusions drawn from the analysis presented above that the agent was introduced into this series by some of the C3H males with which the females were bred, so that some of the females had the agent whereas others did not.

In the C3Hf backcross series (table 6) there was no evidence of a positive parent-offspring correlation in any group. Although the tumor incidence was low in all groups, the tumors occurred just as frequently among offspring of females that did not develop tumors as among offspring of those that developed tumors. Thus, there was no evidence that the mammary tumors arising in this series were the result of the mammary tumor agent.

Litter Seriation

In view of previous reports (*10,11*) that in certain strains or hybrids with the mammary tumor agent a higher incidence of mammary tumors occurs in females born in late litters than in those born in early litters, females of all series were tabulated with respect to litter seriation. The foster-nursed C3Hf females were tabulated with respect to the number of litters the foster mother had borne at the time she foster-nursed the C3Hf females. A large proportion of the females occurred in litters 1, 2, or 3, although some occurred through subsequent litters to litter 7. When the tumor incidences of groups of females of the first litters were compared with those of the corresponding groups of females of combined later litters, no differences in incidence of mammary tumors were observed, nor were such differences observed when the first and second litters combined were compared with combined subsequent litters. Thus, this tabulation failed to give evidence either for or against the presence of the mammary tumor agent in any series. Among offspring of the few C3H backcross females cited above that received the agent from the C3H males, mammary tumors did occur more frequently in females of

TABLE 5.—*Parent-offspring correlation in C3H backcross series*

Generation	Offspring of mothers with tumors						Offspring of mothers without tumors					
	Breeders		Virgins		Foster-nursed virgins		Breeders		Virgins		Foster-nursed virgins	
	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors
C57BL	0	0	0	0	—	—	17	0	0	0		
FN*-C3Hf	—	—	—	—	0	0	—	—	—	—	81	0
(B† × C3H)F_1	0	0	0	0	—	—	17	1	68	0		
FN – C3Hf	—	—	—	—	4	3	—	—	—	—	86	2
(BC3H × C3H)BC_1	0	0	5	4	—	—	19	1	62	0		
FN – C3Hf	—	—	—	—	5	0	—	—	—	—	59	6
(BC3H × C3H)BC_2	0	0	5	0	—	—	17	0	65	1		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	59	4
(BC3H × C3H)BC_3	0	0	0	0	—	—	20	7	36	1		
FN – C3Hf	—	—	—	—	25	14	—	—	—	—	42	1
(BC3H × C3H)BC_4	10	6	12	7	—	—	6	1	36	2		
FN – C3Hf	—	—	—	—	16	15	—	—	—	—	25	4
(BC3H × C3H)BC_5	5	5	16	11	—	—	6	4	11	1		
FN – C3Hf	—	—	—	—	28	21	—	—	—	—	5	1
(BC3H × C3H)BC_6	20	14	14	8	—	—	0	0	0	0		
FN – C3Hf	—	—	—	—	41	29	—	—	—	—	26	14
(BC3H × C3H)BC_7	16	14	52	39	—	—	7	4	16	3		

*FN = Foster-nursed.
†B = C57BL.

TABLE 6.—*Absence of parent-offspring correlation in C3Hf backcross series*

Generation	Offspring of mothers with tumors						Offspring of mothers without tumors					
	Breeders		Virgins		Foster-nursed virgins		Breeders		Virgins		Foster-nursed virgins	
	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors	No.	No. with tumors
C57BL	0	0	0	0	—	—	20	0	0	0		
FN* – C3Hf	—	—	—	—	0	0	—	—	—	—	90	6
(B† × C3Hf) F_1	0	0	0	0	—	—	24	0	74	2		
FN – C3Hf	—	—	—	—	0	0	—	—	—	—	77	1
(BC3Hf × C3Hf) BC_1	0	0	0	0	—	—	19	2	68	1		
FN – C3Hf	—	—	—	—	10	0	—	—	—	—	69	2
(BC3Hf × C3Hf) BC_2	0	0	7	0	—	—	16	1	49	1		
FN – C3Hf	—	—	—	—	3	0	—	—	—	—	58	1
(BC3Hf × C3Hf) BC_3	0	0	0	0	—	—	23	5	29	1		
FN – C3Hf	—	—	—	—	16	1	—	—	—	—	62	0
(BC3Hf × C3Hf) BC_4	4	0	11	0	—	—	15	2	65	2		
FN – C3Hf	—	—	—	—	10	0	—	—	—	—	53	3
(BC3Hf × C3Hf) BC_5	0	0	9	1	—	—	13	4	37	0		
FN – C3Hf	—	—	—	—	19	0	—	—	—	—	20	0
(BC3Hf × C3Hf) BC_6	4	0	5	0	—	—	15	1	16	2		
FN – C3Hf	—	—	—	—	5	0	—	—	—	—	52	1
(BC3Hf × C3Hf) BC_7	0	0	2	0	—	—	23	2	47	1		

*FN = Foster-nursed.
†B = C57BL.

later litters than in those of earlier litters but this was concealed in the over-all tabulation.

Histologic Classification of the Mammary Gland Tumors

As has been emphasized before (*12*), mammary tumors often show great variation in histologic pattern in different areas of the same tumor. Placing a tumor in a particular category, therefore, indicates only that this is the predominant pattern in the microscopic section examined. No attempt is made to divide the tumors into a benign and a malignant group since most of them grow progressively when transplanted, whatever the histologic pattern. We have used the scheme previously described (*13*), which is briefly summarized here. Adenocarcinoma Type A includes tumors composed predominantly of small acini with well-defined lumina forming a glandular pattern. Adenocarcinoma Type B tumors are distinctly of epithelial-cell origin and a glandular pattern is usually obvious, but the structure of these tumors is less uniform than those placed in the Type A group. Cystic dilatations, papillary ingrowths, dilated vascular spaces, and areas of solid cells without luminal spaces are often encountered. Tumors of the Type A and Type B pattern have formed the majority of tumors in mice with the agent. In these mice tumors usually appear before the age of 1 year. A few tumors of the Type A or Type B patterns may be found, however, in mice of advanced ages in which no agent can be detected. Adenocarcinoma Type C tumors usually show the same structure in an entire cross section. The histologic pattern is distinctive and consists of small cysts or clefts lined by a single layer of low columnar epithelial cells closely invested by fusiform cells. The fusiform cells appear to be derived from muscle tissue, since they stain yellow with van Gieson's stain and form no collagen. The fusiform cells may appear singly or in sheets in the intervening stroma, which often appears edematous. With few exceptions, the Type C tumors have been found in old mice without the agent. This form has been especially frequent among certain hybrids (*9,14*). The adenoacanthoma group includes tumors in which the glandular element resembles the Type A or Type B adenocarcinoma, but large areas of these tumors are composed of stratified squamous epithelium. Adenoacanthomas were found frequently in old strain C3Hf mice, without the agent, that had been bred a number of times (*8*). A variant of the adenoacanthoma, called the molluscoid form following Haaland's description (*15*), shows a remarkably organized arrangement, with a central area of keratinization, outwardly radiating epithelial tubules, and peripheral gland formation. Carcinosarcomas show a mixture of malignant epithelial and fibroblastic cells; the epithelial cells often appear elongated and seem to merge with the sarcomatous element. These tumors are frequent in mice without the agent treated with methylcholanthrene (*16,17*). Fibrosarcomas enclosing mammary tissue have been recorded in mice following whole-body irradiation. No tumor of this type appeared in the present experiments. In any large group of mammary tumors from mice, a few tumors are

usually consigned to a miscellaneous group, which includes tumors of unusual morphology that do not fit into any of the preceding categories. Such tumors require individual descriptions.

Because of our familiarity with the types of mammary tumors occurring in strain C3H females, (C3H × B)F_1 hybrid females, and C3Hf females foster-nursed by strain C3H or (C3H × B)F_1's, sections of tumors occurring in these groups were not routinely taken, and thus several of the tumors of these groups were not classified. All other mammary tumors were sectioned and classified except for a few which could not be because of *post-mortem* changes or because the tissue was lost. It is probable that the unclassified tumors in the mice which died early were either Type A or Type B.

The mammary tumors occurring in the various generations of the C57BL backcross series are listed in table 7. In the earlier generations, in which there was a relatively high incidence when the females were relatively young, all tumors but one were listed as either Type A or Type B. In later generations, after the mammary tumor agent had disappeared and when the total incidence of tumors was low and the average tumor age was high, two adenocarcinomas of Type C and one adenoacanthoma were noted. (Since some females had more than one tumor the total number of tumors classified is greater than the number of females with tumors in certain groups in tables 7–10.)

In the C3Hf backcross series that followed the C57BL backcross series a greater variety of tumors was observed than in any other series (table 8). In this series the genetic susceptibility was relatively high but the agent

TABLE 7.—*Mammary tumors in the C57BL backcross series listed according to histologic type*

Generation	No. of females	No. with tumors	Number of females with various types of mammary tumors				
			Adenocarcinoma			Adenoacanthoma	Not classified
			Type A	Type B	Type C		
C3H	20	18	7	4			7
C3Hf FN* on C3H	88	83	37	54			2
(C3H × B†)F_1	90	74	46	29			3
C3Hf FN on F_1	80	55	33	30			1
(C3HB × B)BC_1	82	10	5	5			
C3Hf FN on BC_1	69	54	26	36			1
(C3HB × B)BC_2	73	1	—	1			
C3Hf FN on BC_2	97	18	7	12			
(C3HB × B)BC_3	96	1	—	—	1		
C3Hf FN on BC_3	93	1	—	1			
(C3HB × B)BC_4	84	0					
C3Hf FN on BC_4	89	0					
(C3HB × B)BC_5	87	0					
C3Hf FN on BC_5	76	3	1	1	—	1	
(C3HB × B)BC_6	60	1	—	1			
C3Hf FN on BC_6	89	4	1	1	1	—	1
(C3HB × B)BC_7	78	0					

*FN = Foster-nursed.
†B = C57BL.

TABLE 8.—*Mammary tumors in the C3Hf backcross series that followed the C57BL backcross series, listed according to histologic type*

Generation	No. of females	No. with tumors	Number of females with various types of mammary tumors					
			Adenocarcinoma			Adenoacanthoma	Carcinosarcoma	Miscellaneous
			Type A	Type B	Type C			
$(BBC_7 \times C3Hf)F_1$	100	8	—	1	7			
$(BBC_7C3Hf \times C3Hf)BC_1$	100	7	—	—	3	2	1	1
$(BBC_7C3Hf \times C3Hf)BC_2$	96	3	1	2				
$(BBC_7C3Hf \times C3Hf)BC_3$	100	6	1	4	1	1		
$(BBC_7C3Hf \times C3Hf)BC_4$	103	3	—	2	—	—	1	

was not present. A number of tumors occurred but mostly in old animals. Only two of the tumors in this series were adenocarcinomas, Type A, and only nine were Type B. There were 11 listed as adenocarcinomas, Type C. This was the largest number of this type found in any group, and supports previous observations that this tumor type is relatively frequent in hybrids. There were three adenoacanthomas, two carcinosarcomas, and one miscellaneous tumor.

The mammary tumors found in the C3H backcross series are listed in table 9. In this series few tumors occurred in the early generations, but in later generations, as the susceptible genotype was increased and the milk agent was introduced, the incidence increased and the mammary tumor age decreased. This increase was owing to an increase in adenocarcinomas of Types A and B. The Type C adenocarcinoma occurred in a female of a line that had the agent, but the tumor did not appear until the female was 28 months old. The nine adenoacanthomas in this series did not appear to be directly related to the presence or absence of the agent. They occurred in both early and late generations; four in the later generations were in females of lines in which the agent was definitely present. The other five were in females that probably did not have the agent. All 9 adenoacanthomas occurred in animals past 10 months, the average age being over 20 months. The carcinosarcoma occurred in a female 10 months of age in a line with the agent.

The mammary tumors, occurring in the C3Hf backcross series, are listed according to histologic type in table 10. Owing to the high genetic susceptibility of the females in the generations beyond the original C57BL females, some tumors appeared but the incidence in the various generations remained relatively low and the average tumor age remained relatively high. In this series the mammary tumors were approximately equally divided between adenoacanthomas and each of the three types of adenocarcinomas—A, B, and C.

Discussion

This study extends our earlier observations that in our strains C3H and C57BL and their hybrid descendants the mammary tumor agent survives

TABLE 9.—*Mammary tumors in the C3H backcross series listed according to histologic type*

Generation	No. of females	No. with tumors	Number of females with various types of mammary tumors						
			Adenocarcinoma			Adeno-acanthoma	Carcino-sarcoma	Miscel-laneous	Not classi-fied
			Type A	Type B	Type C				
C57BL	17	0							
C3Hf FN* on B†	81	0							
(B × C3H)F_1	85	1	—	1					
C3Hf FN on F_1	90	5	3	2					
(BC3H × C3H)BC_1	86	5	1	2	—	1			1
C3Hf FN on BC_1	64	6	3	2	—	1			
(BC3H × C3H)BC_2	87	1	—	—	—	1			
C3Hf FN on BC_2	59	4	2	1	1				
(BC3H × C3H)BC_3	56	8	2	4	—	2			
C3Hf FN on BC_3	67	15	12	2					1
(BC3H × C3H)BC_4	64	16	9	7				1	
C3Hf FN on BC_4	41	19	13	6	—	1			1
(BC3H × C3H)BC_5	38	21	11	11					1
C3Hf FN on BC_5	33	22	10	13	—	1	1		
(BC3H × C3H)BC_6	34	22	10	12	—	1			1
C3Hf FN on BC_6	67	43	30	14	—	1			
(BC3H × C3H)BC_7	91	60	34	28					1

*FN = Foster-nursed.
†B = C57BL.

TABLE 10.—*Mammary tumors in the C3Hf backcross series listed according to histologic types*

Generation	No. of females	No. with tumors	Number of females with various types of mammary tumors					
			Adenocarcinoma			Adenoacanthoma	Miscellaneous	Not classified
			Type A	Type B	Type C			
C57BL	20	0						
C3Hf FN* on B†	90	6	3	2	1	—	1	
(B × C3Hf)F_1	98	2	—	—	2			
C3Hf FN on F_1	77	1	—	—	1			
(BC3Hf × C3Hf)BC_1	87	3	—	1	1			1
C3Hf FN on BC_1	79	2	—	2				
(BC3Hf × C3Hf)BC_2	72	2	—	—	—	2		
C3Hf FN on BC_2	61	1	1					
(BC3Hf × C3Hf)BC_3	52	6	2	3	1			
C3Hf FN on BC_3	79	1	—	1				
(BC3Hf × C3Hf)BC_4	95	4	2	—	1	1		
C3Hf FN on BC_4	63	3	1	—	1	1		
(BC3Hf × C3Hf)BC_5	59	5	1	1	1	2		
C3Hf FN on BC_5	39	0						
(BC3Hf × C3Hf)BC_6	40	3	—	1	—	2		
C3Hf FN on BC_6	57	1	—	1				
(BC3Hf × C3Hf)BC_7	72	4	2	1	—	1		

*FN = Foster-nursed.
†B = C57BL.

only when associated with the proper gene or genes. The variation observed in the backcross of the earlier work suggested that the genes involved probably were multiple in number with additive effects. In the present study, however, the rapidity with which the agent was completely eliminated in the C57BL backcross series would call for some modification of the earlier interpretation. The fact that the agent was completely eliminated by the third backcross would indicate that the genes were few in number and would even suggest the possibility of but a single gene difference in this respect between these two strains. Even the earlier data would not contradict a single-gene interpretation if the lapse of one generation is required for the agent to die out in the absence of the gene. Such a concept finds support in the observations of Andervont (*3*) that C57BL females of this subline that received the agent could themselves transmit it but their C57BL female offspring could not. This might also be compared with the "cytoplasmic lag" noted particularly in lower organisms or in the lag through several cell generations in the disappearance of *kappa* in the absence of the gene in *Paramecium* (*18*). A project based on the assumption that one generation may be required for the eradication of the mammary tumor agent in the absence of the gene is now under way and it is hoped that in this study it will be possible to distinguish between single- and multiple-gene segregation.

The present study sheds some light on the questions of latency and whether or not the agent can exist in a "masked" or inactive state. Such questions are confused if one considers the presence or absence of a tumor in an animal as evidence either for or against the presence of the agent.

There is now ample evidence that not all mammary tumors of the mouse are associated with the agent or that not all mice with the agent develop tumors. The presence of the agent merely increases the probability that a tumor will occur and its absence decreases this probability. At the present time probably the best evidence that the agent is present in a female is a relatively high incidence of tumors at a relatively early age in her susceptible descendants or in her susceptible foster-nursed test females. In this study there were ample test females for each breeding female, or ample susceptible female descendants, so that one could be rather sure whether or not that female had the agent. With such a test system there was no evidence at any time throughout the study that the agent remained inactive over a number of generations and then suddenly reappeared. When the agent disappeared because of the absence of the proper gene or genes, it had not merely become inactive, to reappear when the proper genes were reintroduced. When the agent was introduced, even in presumably very small quantities from the males, one could not only identify the females that became infected, but also it was obvious at which mating she became infected, for her own female offspring or her foster-nursed females following this mating clearly had the agent.

The present study failed to support the concept of a possible intrinsic origin of the agent or a *de novo* appearance of the agent. The agent did not arise in either of the two C3Hf backcross series, although the females of both series were highly susceptible to it. It has not arisen *de novo* in the 29 generations of our C3Hf line which, of course, is highly susceptible to it. It did arise in three females of the C3H backcross series, but in these cases it was obvious that the females were infected by the C3H males with which they mated.

This observation of the transmission of the agent by the male is in line with similar observations that have now been published by a number of other investigators (*10,19–21*). It should be emphasized, however, that in the present study transmission by the male was of rare occurrence, but when a female was infected by the male she then infected her offspring, and in her genetically susceptible descendants a high-tumor line immediately appeared.

With the development of lines in which mammary tumors occur in the absence of the agent both spontaneously and induced with carcinogenic hydrocarbons, there has been renewed interest in the histologic types of mammary tumors. Attempts have been made to ascertain whether or not certain types are associated with presence or absence of the mammary tumor agent. Several histologic studies with a review of large numbers of mammary tumors have recently been reported from other laboratories. Dmochowski (*22*) examined 377 mammary tumors in C57 × RIII hybrid mice and 141 in pure strain RIII mice. He reported no correlation between the appearance of a tumor and its location, size, rate of growth, litter sequence, or age, except in "squamous type tumors," which as a rule developed in older mice above the age of 12 months. Mühlbock *et al.* (*23*) reviewed the morphology of mammary tumors in

strain DBA mice with and without the agent. He found that ". . . DBAb mice without the agent show a wider variety of histologic pictures, and the percentage of tumors with an acinar solid structure and its varieties is not so predominant as in animals with the agent." It was noted further that ". . . the absolute number of carcinomas having a variable form in DBA with the agent (a few of a large number of tumors) differs little from that in DBA without the agent (many of a large number of tumors)." Earlier, Kirschbaum (*24*) had found that a relatively high proportion of mammary tumors in mice without the agent displayed squamous metaplasia but considered it as indicative of development independent of the milk agent. In a series of mammary gland tumors that Dmochowski (*22*) tested for the agent one classified as an adenoacanthoma had the agent.

When one considers all series of the present study it is apparent that the adenocarcinomas of Type A and Type B occurred most frequently in younger females with the mammary tumor agent. These types, however, were not limited to the younger females nor to those with the agent. The adenocarcinomas of Type C and the adenoacanthomas tended to occur more frequently in older females without the agent. It may be that the involutionary changes of aging play an important part in determining these unusual tumor types and that the absence of the agent was associated with these types only because the tumors arose in agent-free females at a more advanced age. In the C3H backcross series some adenoacanthomas occurred in females that must have had the agent, as did the adenocarcinoma of Type C in this series. Since few females with the agent survive to advanced age, no large group of mammary tumors in old mice with the agent is available for comparison.

In these series of females, many of which lived to relatively old age, a wide variety of tumors other than of the mammary gland occurred. A tabulation of these along with descriptions of those not previously described will be given in a later publication.

Summary

Strain C3H female mice with the mammary tumor agent were outcrossed to strain C57BL males without the agent and genetically resistant to it, and the female offspring were backcrossed to strain C57BL males through seven successive backcross generations. By the third backcross generation the agent had been eliminated by concentration of strain C57BL chromatin. The fact that the agent was eliminated so early would suggest that these strains differed by only a few genes controlling the propagation and transmission of the agent and possibly by only a single pair of genes.

Females of this seventh backcross generation were outcrossed to C3Hf males (without the agent) and their female progeny backcrossed to C3Hf males through four backcross generations. The mammary tumor agent did not reappear with the concentration of C3H chromatin, indicating that the concentration of C57BL chromatin had eliminated the agent rather than causing it to mutate to a latent or inactive form.

Genetically resistant C57BL females without the agent were outcrossed to genetically susceptible C3Hf males without the agent, and the female offspring were backcrossed to C3Hf males through seven backcross generations. The mammary tumor agent did not appear in these generations, thus failing to give evidence of an intrinsic or of a *de novo* origin of the agent.

C57BL females without the agent were outcrossed to C3H males with the agent and the female offspring were backcrossed to C3H males through seven backcross generations. The agent was transmitted to certain of the females from the males and propagated in their offspring, building up certain lines with mammary tumor incidences comparable with those of the strain C3H.

The mammary tumors occurring at an early age in the females with the mammary tumor agent were for the most part adenocarcinomas of Types A and B. Some tumors of these types did, however, occur in older females with and without the agent. Adenocarcinomas of Type C and adenoacanthomas occurred mostly in females of an advanced age both with and without the agent. It may be that their occurrence is related to histologic changes of the mammary gland with advancing age rather than directly related to the absence of the agent.

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Analysis of Variability of Mammary Tumor Incidence in Inbred DBA Mice [1, 2]

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Since the establishment of inbred strains of mice differing in susceptibility to mammary tumor, experiments (*1,2*) involving crossing and fostering between low- and high-tumor-incidence strains to observe the tumor incidence of their offspring have contributed greatly to understanding of mammary tumor inheritance. It is clear that most of these studies were limited to crosses between strains, with each strain regarded as a single unit. Consideration of the pattern of incidence of cancer *within* a large, relatively homogeneous population has largely been omitted from such studies, although its analysis, added to comparison of interstrain variations might contribute greatly to understanding the manner of contribution of factors known to have a strong influence on mammary tumor incidence, such as genes, hormones, and the mammary tumor inciter or milk agent. The analysis may also disclose significance of certain environmental factors and evaluate their relative importance in cancer development.

In this paper we will analyze variability of mammary tumor incidence in inbred DBA mice. The study is based on life-history and pathology records accumulated from 1947 to 1953 for several sublines of the DBA strain mice maintained in the Jackson Laboratory Inbred Nucleus. DBA mice are particularly suitable for causal analysis of mammary tumor incidence because in all substrains the incidence falls slightly above the 50 percent level. At this sensitive level, a slight shift in underlying causes leads to an easily discernible change in tumor incidence. Analysis included selection of a basis for evaluation, characterization of the population as a whole, and analysis of variations in incidence associated with division of the population into subgroups based on a number of criteria. This last procedure established the degree of determination of differences in incidence by genetic differences between sublines, the extent of correlation between relatives and its probable meaning in each subline, and the extent and nature of effects of environmental factors which could be analyzed

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from breeding records (seasons, position in parents' litter seriation, number of litters produced). All of these analyses have been integrated to present their total impact upon understanding of the factors involved in mammary tumor genesis.

General Description of Material Analyzed

Six colonies of DBA mice have been maintained in the Inbred Nucleus of the Jackson Laboratory since 1948. Before that time they had quite distinct histories. Two of the sublines, DBA/2HeJax and DBA/1Jax, are maintained in considerably larger numbers than the others, since they are the immediate ancestors of very large breeding colonies used for both research within the laboratory and supply. The other four have been maintained in small numbers for purposes of comparison. The first of the large sublines, DBA/2HeJax, stems from a full-sib mating in the 31st generation of brother × sister mating obtained from the colony of Dr. W. E. Heston of the National Cancer Institute. The second large subline is DBA/1(40)Jax, derived from a very few nonpedigreed survivors of a pre-fire (1947) DBA/1 colony salvaged by Dr. K. P. Hummel and interbred, although they were not brother × sister pairs. Although these survivors descended originally from the DBA/1 line maintained and studied by Dr. Elizabeth Fekete, it is impossible to state exactly the last generation at which they had a common ancestor, except that it was long after the original inbreeding of the strain. The first mating of this family in the Nucleus colony was recorded as ♀40 × ♂39, hence the (40) designation. Due to the interruption of brother × sister mating, and consequent possible opportunity for heterogeneity, the generation number of this pair was recorded as 3 (interrupted; previously 20+). Due to the long previous history of inbreeding, the number of heterozygous gene pairs affecting mammary tumor incidence in DBA/1(40) must, however, be very small compared to the number of homozygous gene pairs influencing this character. The four minor sublines include: DBA/1(1)Jax, from a brother × sister mating in the colony of Dr. Jean Brandenburg, Department of Radiology, University of California; DBA/MuJax and DBA/WaJax, both originating from brother × sister matings in the colony of Dr. William S. Murray, then at the Detroit Cancer Institute; and DBA/2ViJax, from the colony of Miss Emilia Vicari at the Hamilton Station of the Jackson Laboratory. For additional data and generalization, smaller numbers of animals from these four presumably homogeneous colonies were incorporated in certain determinations, as indicated under the pertinent analyses. Thus these studies are mostly based on one large, possibly heterogeneous colony of DBA/1 and one large, presumably homogeneous colony of DBA/2, with additional data in some cases from minor sublines. It should be remembered that DBA/1 and DBA/2 have been shown repeatedly to have a different average tumor incidence and to resist each other's transplantable tumors, indicating a considerable degree of genetic difference between them.

The total number of breeding females in the 6 substrains of DBA mice

covered in this study was 517. All animals had survived more than 200 days—those dying before this age were rejected. A few females which produced no offspring, although they were constantly with a male, have been included. This inclusion seems advisable to broaden the base of generalization on effect of litter production.

The grand mean tumor incidence was 64 percent, varying from 56 percent in DBA/2He to 72 percent in DBA/1(40). The number and percentage of tumor-bearing animals in each group are given in table 1. The age at which tumor occurred varied from 182 to 629 days. There were some differences among the substrains in life span and age at occurrence of tumor. In table 1 the column on life span refers to the average age of animals dying without tumor. The mean tumor age column refers to time of first appearance of tumor rather than to time of death, which in DBA mice usually occurs approximately 1 to 2 months later.

TABLE 1.—*Tumor incidence with tumor age and non-tumor life span*

Substrains	Tumorous animals		Nontumorous animals		Tumor incidence
	Number	Mean age at first tumor (*days*)	Number	Mean life span (*days*)	(*percent*)
DBA/1(40)Jax	102	356	39	401	72
DBA/2HeJax	102	401	81	405	56
Other minor substrains*	125	390	68	438	65
Total	329	383	188	416	64

*Other minor substrains include DBA/1(1), DBA/Mu, DBA/Wa, and DBA/2Vi.

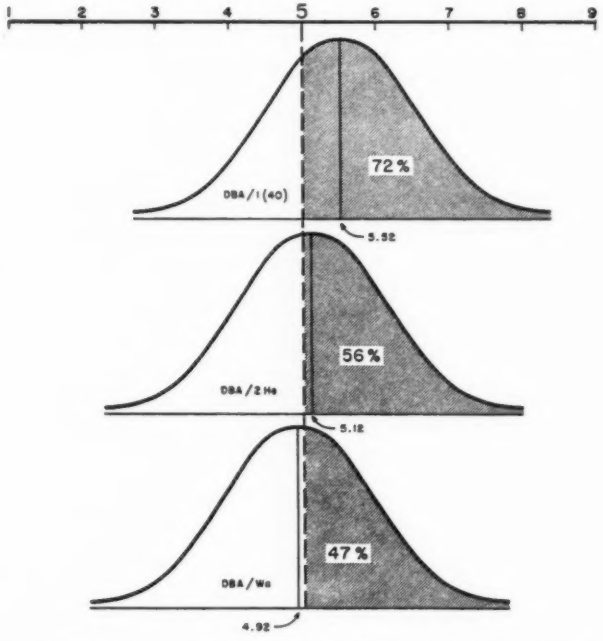
Degree of Determination by Heredity

Heston (*3*) has suggested that the appearance of mammary tumors may be considered as a threshold character affected by many genes interacting with the environment. A wide variety of these characters have been investigated in many inbred strains and interstrain hybrids from several species of mammals. Mammary tumor differs, however, from most of the other characters studied in this way in that it appears very late in the life span of affected animals. Most characters previously studied have been determined before birth or during early postnatal growth, as for example the number of presacral vertebrae or the incidence of cleft palate. The environmental effects which have contributed to variation observed in these previously investigated cases have thus been largely either intra-uterine or nursing effects. In a late-appearing character such as mammary cancer, many more environmental factors have had an opportunity to affect expression of the character, making the situation more complex. It is to be expected that only very strong effects of the early environment will persist, in spite of possible dilution by later influences, to produce significant differences at the end of the life

span. There is also, of course, much more opportunity for later-acting environmental factors, such as diet, season, and breeding activity, to have an effect.

In the analysis of threshold characters it may be assumed as a working hypothesis that there is in each strain a group of genetic factors, each of which makes a constant contribution to the level of mammary-tumor-favoring influences. The effect of these factors, fixed in each strain, may be considered to determine a mean level. The varying environmental influences add to or substract from this value and are assumed to be distributed normally about the mean. Mammary tumors will appear only in that portion of the distribution where the combined effect of genes and environmental influences falls above a certain critical threshold.

The relative distributions of the two major substrains and the largest minor substrain (DBA/Wa) are illustrated diagrammatically in text-figure 1. Since the only classification which can be made is the dichotomy of tumor *versus* non-tumor, the relative positions of the means were determined from the percentage of tumor incidence in each substrain, applying the inverse probability function. The threshold value has been set arbitrarily at 5 on the chart. It is easy to see from this diagram that the distributions of the three substrains are close to each other. The



TEXT-FIGURE 1.—A diagram to illustrate the relative distribution of two major substrains and the largest minor substrain with reference to the threshold value, which is arbitrarily set at 5 on the scale. The means are 5.52 for DBA/1(40), 5.12 for DBA/2He, and 4.92 for DBA/Wa.

mean of DBA/Wa is slightly below the threshold limit, and the two other substrain means are above it. None of the differences between means are as great as one standard deviation, indicating that differences in genetic level behind the observed differences in tumor incidence need not be very extensive.

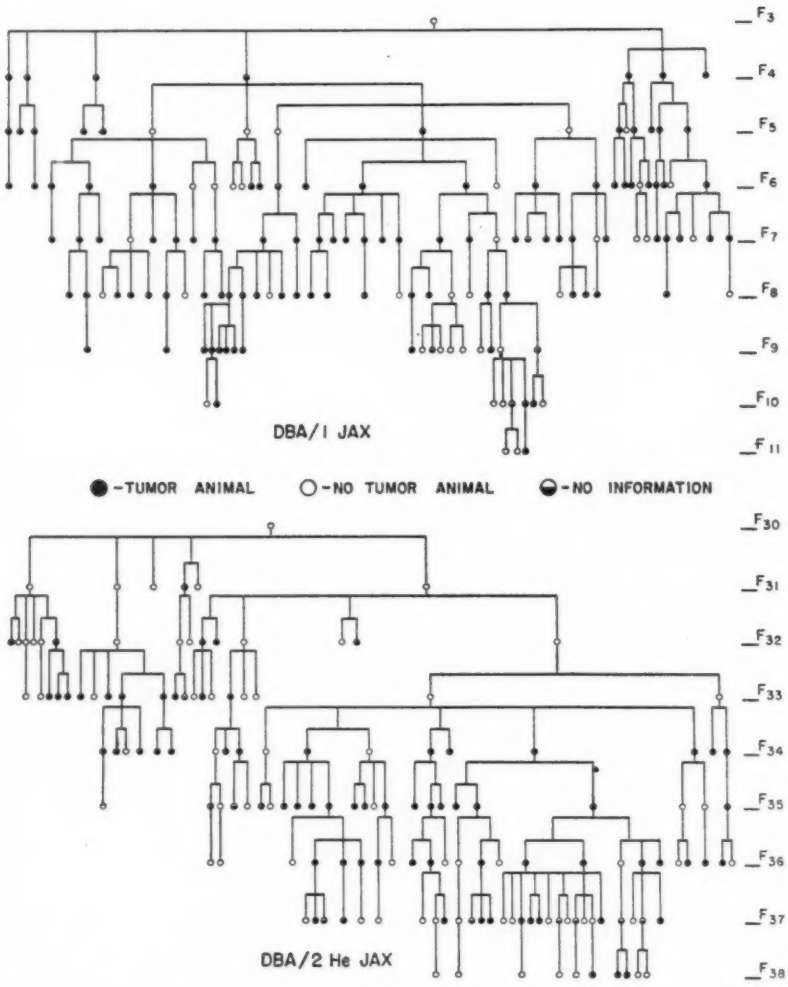
The genetic contribution to the total variation of mammary tumor incidence among the substrains was estimated to be about 10 percent. The computation was done according to the method of inverse probability given by Wright (*4*), assigning unit standard deviation to each substrain. It may be desirable to point out that the possible heterogeneity of the DBA/1(40) substrain should cause no complication, because each substrain was considered as a single unit and the standard deviation in each was assigned as unit.

Correlation between Relatives with Reference to Tumor Incidence

Correlation with reference to tumor incidence between relatives within each substrain may give some information about the relative importance of the genetic influences in comparison with the nongenetic influences on mammary tumor genesis. Dam-daughter, littermate, and sibling correlations were computed. The two major substrains, DBA/1(40) and DBA/2He, were analyzed separately. The other minor substrains were omitted from separate analysis because of small sample sizes but were included in a pooled analysis, which covered all substrains.

Plots to show the relationship of mammary tumor incidence among relatives should be a preliminary procedure in correlation analysis. However, this cannot be done in the present case because the incidence is a character of dichotomy. Instead, a sketch of the tumorous and nontumorous animals in a pedigree form for the family of each major substrain is presented in text-figure 2, illustrating the distribution of tumorous animals among family branches as well as closely related individuals. In the smaller substrains, distributions are quite similar to those of the DBA/2He family except for differences in total incidence. Examining the pedigree diagram, the occurrence of tumors appears to be scattered in all branches of the DBA/2He family; there is no tendency for the tumorous individuals to cluster more heavily in certain branches than in others. In the DBA/1(40) family, however, there is a slight difference in tumor distribution among different branches. In addition, there is an excess over expectation of litters that are either all tumorous or all nontumorous. These suggest the possibility of segregation of genetic factors and existence of correlation among relatives.

It should be noticed also in the pedigree chart that the tumor incidence in the progenies of tumor-bearing ancestors is not always higher than that of the non-tumor-bearing ancestors. For instance, the 7th individual from the right side in the 5th generation of the DBA/1(40) family had no tumor and lived 366 days. Of her 13 female progeny, 11, or about 84 percent, were tumorous. By contrast, one of her litter mates, also a nontumorous individual, lived 465 days and 15 of her 17 female descend-



TEXT-FIGURE 2.—Pedigrees of family 40 of DBA/1Jax and one family of DBA/2HeJax.

ants, or about 88 percent, were tumorous. These may be compared with the third litter mate, which developed a mammary tumor and produced 39 offspring of which 16, or 59 percent, had tumors. Similar situations occurred in other substrains. These indicate strong environmental influences on the variation in expression of the character, and no other information contradicts this observation.

A brief discussion of the methods of correlation analysis may be helpful. As mentioned previously, the character in question is a dichotomy of tumor and lack of tumor. The method of coefficient of mean square contingency (*5*) would be a convenient one to apply, but it is limited in that

the maximum value varies with the number of cells in the table. The χ^2 value with only a single degree of freedom would be an approximation and would need correction for discontinuity. On the other hand, the method of tetrachoric correlation would be satisfactory if the total sample were large enough and if the percentage of the total occurring in each cell were larger than 0.05. These limitations for tetrachoric analysis are met in these data. Furthermore, the correlation coefficient obtained from tetrachoric analysis has been widely used by biologists, and the values obtained are easy to evaluate. Therefore, the latter method has been applied as an appropriate test. The coefficients of tetrachoric correlation, r_{tet}, were obtained by using the computing diagrams prepared by L. L. Thurstone and others (*6*), and the standard errors were computed according to Kelley (*7*).

Correlation between Dam and Daughter

The tendency for a tumorous dam to produce an excess of tumorous daughters and for a nontumorous dam to produce an excess of nontumorous daughters varied among the substrains (table 2). This tendency was in general very slight except in DBA/1(40), which had a strong tendency toward such arrangement. In DBA/2He this distribution seemed on inspection to be random. The apparent nonrandom distribution in the combined minor substrains probably resulted from substrain differences in tumor incidence.

TABLE 2.—*Distribution of dam-daughter pairs with reference to tumor incidence*

Substrains	Tumorous dam with		Nontumorous dam with		Total number of pairs
	Tumorous daughter	Non-tumorous daughter	Tumorous daughter	Non-tumorous daughter	
DBA/1(40)	87	16	21	12	136
DBA/2HeJax	56	41	33	26	156
Other minor substrains	71	30	27	27	155
Total	214	87	81	65	447

The coefficients of correlation between dam and daughter with respect to tumor incidence were computed for each substrain and are given in table 3. The correlation value is significant in DBA/1(40) and clearly nonsignificant in DBA/2He. The only possible contribution to this difference seems to be from a genetic source. Since the DBA/1(40) line is descended in a recent generation from an unrelated pair of mice, there is a high probability that minor genetic segregation is in process, with consequent slight differences in the genes fixed in different portions of the pedigree. In DBA/2He, with no interruption of inbreeding, evidence indicates that a high degree of genetic homogeneity was maintained. No dam-daughter correlation would be expected in this case unless there were nongenetic

TABLE 3.—*Coefficients of tetrachoric correlation (r_{tet}) for dam-daughter litter mates and siblings*

Substrains	Dam-daughter		Litter mates		Siblings	
	No. of pairs	r_{tet}	No. of pairs	r_{tet}	No. of pairs	r_{tet}
DBA/1(40).....	136	.38±.04	37	.52±.08	111	.22±.05
DBA/2He.....	156	.05±.05	54	.24±.08	165	.01±.05
All substrains....	447	.25±.03	147	.36±.05	387	.14±.03

effects common in both dam and daughter. The following analysis (litter mates) may give further evidence to support this interpretation.

In the calculation of the dam-daughter correlation, in order to obtain as much information as possible from this limited amount of data, all possible dam-daughter pairs were entered into the analysis. Therefore both concordant and discordant dam-daughter pairs were included, in addition to the repetition of those dams with more than one female offspring. Since each pair is treated as independent, testing the null hypothesis that there is no real relationship, this inclusion should cause no complication (E. M. Dempster and Howard Levene, personal communication).

Correlation between Litter Mates

Some tangible environmental factors act similarly on litter mates. If these factors play an important role in the causation of mammary tumor genesis, there should be a tendency for litters to consist predominantly of tumorous or nontumorous animals. By comparing the observed and calculated frequencies of litters with different combinations of tumorous and nontumorous individuals, a test may be made of the hypothesis of random occurrence of tumorous individuals in a litter. Table 4 gives both the observed and calculated frequencies of litters containing only two females. The incidence of tumors in individuals from such litters was

TABLE 4.—*Observed and calculated frequencies of tumor incidence for litter mates in the two major substrains*

Strain	Size of litter	No. of tumorous individuals in litters						No. of litters	No. of young	Tumor incidence (*percent*)
		0	1	2	3	4	5			
DBA/1(40)	2	obs. 5	6	14				25	50	68.0
		calc. 2.56	10.88	11.56						
		diff. +2.44	−4.88	2.44						
	3	obs. 0	1	1	2					
DBA/2He	2	obs. 6	13	13				32	64	60.9
		calc. 4.88	15.23	11.88						
		diff. +1.22	−2.23	+1.12						
	3	obs. 0	1	2	1					
	5	obs. 0	0	0	0	0	1			

68.0 percent in DBA/1(40) and 60.9 percent in DBA/2He. The expected frequencies are calculated according to the binomial expansion of $(.680T+.320N)^2$ in the former subline and according to $(.609T+.391N)^2$ in the latter. No frequencies were calculated for litters with 3 to 5 females because the number of such litters was too small.

Comparing the observed and calculated values in the two major substrains, an excess of either wholly tumorous or wholly nontumorous litters and a deficiency of mixed litters is found. In DBA/1(40) these discrepancies are significant ($P<.02$). Although the differences in DBA/2He are not statistically significant, they are in the same direction as those in DBA/1(40), differing only in magnitude. These also suggest that a relationship exists among litter mates in reference to mammary tumor incidence.

The computed coefficients of correlation among litter mates were found to be significant in both DBA/1(40) and DBA/2He substrains. It is interesting to see that littermate correlation is higher than dam-daughter correlation in each major substrain. Comparison of substrains shows both littermate correlation and dam-daughter correlation to be higher in DBA/1(40) than in DBA/2He. This seems to be a second evidence of the presence of a genetic component in the estimate of correlation for DBA/1(40), in addition to the existence of environmental influence common to litter mates in both substrains.

Correlation between Individuals from the Same Mating

The coefficients of sib correlation were computed and given in table 3. They are, in general, lower than the dam-daughter correlations of the respective substrains, but the differences may not be significant. This indicates that environment common to individuals from the same mating does not contribute significantly to tumor incidence.

Mention should be made of the computation of the correlation coefficients among either litter mates or sibs. Since the sizes of litters and of sib groups varied within substrain, weighted averages could be computed according to Wright (*4*). However there is no reason to suppose that a different weighting for different sizes of litter or sib groups would necessarily be any better than equal weighting for each pair (Dempster and Levene, personal communication). In fact, there were only a few litters with more than two females entered in the computation of littermate correlation. Should any bias result from the equal weighting, it would be of very small magnitude and could be ignored.

We may summarize briefly the correlation analysis between relatives with reference to mammary tumor incidence. The correlation values of DBA/1(40) are higher than all the others, while littermate correlations are higher than either dam-daughter or sib correlations in each substrain and in the total. The differences between correlations of dam-daughter and sibs, although in the same direction, are not significant. In DBA/2He both are practically nonexistent.

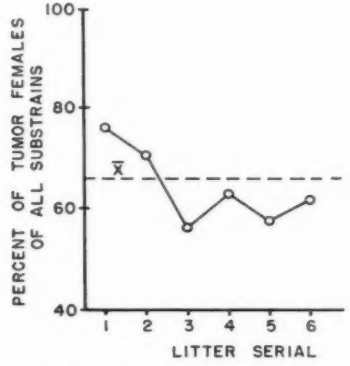
Environmental Effects on Tumor Incidence

Seasonal effects.—The number of tumors appearing in different months was tabulated. The date of first tumor was taken if more than one tumor appeared. Although there are some seasonal fluctuations in tumor incidence there is no significant trend toward monthly variation. Similarly, a classification of tumor incidence of females arranged according to the month in which they were born shows no significant differences. These data seem to indicate that seasonal difference contributes no effect on mammary tumor potentialities either of adults or newborn young.

Litter size and litter-seriation effects.—Since in these data the number of weanling mice in a litter is directly proportional to the number of newborns in the same litter, either value may be taken for the analysis of effect of litter size. The number of weanling young was taken for this analysis. The number of tumorous and nontumorous animals in litters of one to eight were tabulated. There are no significant differences in the percentage of tumorous animals from litters of different sizes.

An effect of serial order of litters on tumor incidence has been reported (7). It would be interesting to see if this relationship holds for the present data. All tumorous and nontumorous animals were grouped together according to litter seriation and the percentage of tumor incidence computed (text-fig. 3). Approximately 80 percent of the animals were produced from the first to third litters, although there were some differences among the substrains.

There appears to be a general tendency for more tumorous animals in the first and second than in later litters, although a few high values occur after the second litter in some substrains. To test the litter serial effect the pooled results from all substrains would be most dependable (text-fig. 3). The total values indicate that animals from first and second litters had tumor incidences above 70 percent, while those from litters 3 to 6 were below 63 percent. The results of the χ^2 test for the total of each litter serial and for the comparison of the first two litters against the re-



TEXT-FIGURE 3.—Relationship of tumor incidence and sequence of litters to which females belonged. $\bar{X}$ is the average percentage of tumorous animals.

mainder both give probability values about the 25 percent level. The computed coefficient of regression of tumor incidence on litter seriation with weight attached also turned out to be insignificant ($b=.026\pm.0147$, $0.1<P<0.2$). Thus no definite conclusions can be made from this test alone. Nevertheless, it may be safe to say that litter seriation did not account for more than 4 percent of the total variance.

This result differs from that of Bittner (*8*) in which animals from the first and second litters had lower tumor incidence than those from later litters. One difference is that Bittner's data were based on hybrid animals, whereas these are from pure, inbred strains. Differences in colony management leading to differing intervals between litters might also contribute. It is difficult to conclude without more data whether different genetic constitutions would behave differently in this respect.

Effect due to reproduction.—It is well known that the reproductive activity of females affects their tendency to mammary tumor. In this respect strains of mice behave differently. In some strains virgin females have much lower tumor incidence than breeders. In the A strain the incidence of mammary tumor in the breeding females is 73 percent while that of virgins is less than 5 percent (*3*). Jones (*9*) demonstrated an increase in tumor percentage directly proportional to the number of litters produced by breeding females of the A strain. In other mammary tumor strains the differences are less dramatic.

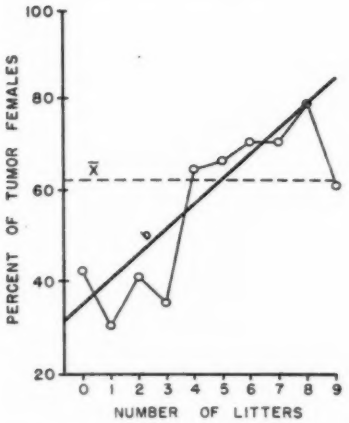
It would be interesting to see how and to what extent this factor affects tumor incidence in DBA mice. Tumorous and nontumorous females of all substrains were tabulated according to the number of litters produced ranging from zero to nine (table 5). Females with zero litters were actually mated, rather than virgin. Although close to 10 percent of the females gave no offspring, the modal value was five or six litters. There appears to be a general tendency for tumor incidence to increase with increase in number of litters produced. A test of this relationship was applied, combining tumorous and nontumorous females of all substrains. A significant regression ($P<.001$) of tumor incidence for number of litters produced was found (text-fig. 4).

The number of litters produced by a female accounts for 13 percent of the total variance of tumor incidence.

It was first thought that the increase of tumor incidence with number of litters might be a nonlinear rather than a linear relationship, that is, the increase of tumor incidence might level off gradually as the number of litters became very large. However, the linear-correlation analysis gave a coefficient of .81. This high value means that the linear relationship can be considered as a rather good fit to these data. The regression coefficient gave a value of 4.5 percent. Thus each additional litter produced increased the tendency to tumor by approximately 5 percent. A graph to show this relationship is given in text-figure 4.

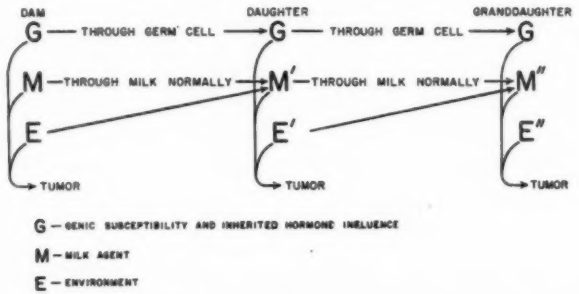
Discussion

A diagram illustrating possible environmental and genetic relationships in mammary tumor genesis and the manner of its transmission in mice



TEXT-FIGURE 4.—Relationship between tumor incidence (ordinate) and total number of litters produced (abscissa) for all substrains combined. $\bar{X}$ is the weighted average of tumor incidence. $b(=.046)$ is the slope of the regression line.

has been constructed (text-fig. 5), indicating that genetically determined hormonal influence and genic susceptibility are transmitted generation after generation without change, while the milk agent may change slightly. Environmental factors may interact with either type of inherited influence to alter the exact level of hormones or susceptibility present in a particular individual. Of course, environment may also cause a certain amount of variation in the milk-agent level among individuals. The milk-agent concentration may depend upon the amount received from the mother. There is also evidence (*10*) that the milk agent may be dependent on the genotype of the animal carrying it for survival and/or proliferation. To avoid confusion, these complicated interactions have been omitted from text-figure 5. It seems to us that this diagram, including the relationships between generations, may give a reasonable picture of factor action and interaction.



TEXT-FIGURE 5.—A diagram to illustrate mammary tumor genesis and transmission in strain DBA mice.

Vol. 16, No. 6, June 1956
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TABLE 5.—*Relationship between tumor incidence and number of litters produced*

Substrains	Number of litters																					
	0		1		2		3		4		5		6		7		8		9		Total	
	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.	No.	%tu.
DBA/1(40).....	9	56	3	33	3	33	2	50	8	75	27	74	23	87	28	71	25	88	12	75	140	75
DBA/2He.....	18	28	6	17	19	37	18	33	25	72	28	64	24	50	23	65	16	75	7	57	184	53
Other minor substrains.....	15	70	4	50	14	50	14	36	35	57	30	63	37	77	25	76	17	71	2	0	193	62
Total.....	42	42	13	31	36	41	33	35	68	65	85	67	84	71	78	71	58	79	21	61	517	62

The importance of genetic contributions to the differences between strains of mice in mammary tumor incidence has previously been demonstrated by outcrossing C3H females to C57BL males and backcrossing the F_1 females to each parent strain (*11*). Animals genetically of low susceptibility to mammary tumor give very little response to either hormone or milk-agent stimulation, while those of high genetic susceptibility are very responsive. Genes for tumor susceptibility should remain the same in the individuals within a highly inbred population unless mutation occurs. Variation between substrains in these data, which must be due mainly to genic difference in susceptibility and hormonal influence, account for 10 percent of total variation.

The significant dam-daughter correlation in DBA/1(40) substrain is interesting. As in a randomly bred population, two times the dam-daughter correlation is an estimate of heritability. The correlation value (0.38) which we obtained suggests a high heritability of mammary tumor incidence, although we cannot apply this value directly for the estimation without correction for inbreeding. This indicates that genetic influence on tumor incidence in mice is even stronger than that on some of the other quantitative characters, such as egg and milk production in farm animals, for which the estimate of heritability is about 0.05 to 0.55 percent. Actually, in view of the wide difference among mouse strains in mammary tumor incidence, this outcome is not surprising. Consequently, these results would be additional evidence from an intra-substrain source to support the hypothesis of genetic susceptibility to tumor incidence in mice.

The results indicate further that the DBA/1 animals in the pre-fire colony probably were not all genetically identical, although they descended from the same substrain and had had a long history of close inbreeding. The pair of unrelated animals, from which family 40 descended, might not differ by many genes. However, some genetic factors affecting tumor incidence seem to be still in the process of segregation in this family up to the time of this analysis. On the other hand, the absence of parent-offspring and sibling correlation in DBA/2He indicates that genetic control of tumor incidence in this subline is in a static state, with the animals maintaining a homozygous condition with regard to genes affecting susceptibility to mammary cancer.

Since the genetic correlations between parent and offspring and among full siblings are theoretically the same, the high littermate correlation obtained at present, exceeding either dam-daughter or sibling correlation, must be contributed from environmental factors. It may be that part of the correlation between litter mates is due to variation in milk-agent concentration. Individuals of one litter would receive approximately the same concentration of milk agent, which may be higher or lower than that received by individuals of other litters from the same parent. Aging of the uterus is another possiblity for explaining such variations, but this effect is, in any case, small since litter-seriation effect contributes only about 4 percent to the total variance. The main part of the correlation

must be due to environmental modification interacting with genic susceptibility and/or hormonal influence before birth or at least before weaning. Such effects would be common to litter mates.

It has been suggested that hormonal influences upon mammary tumor incidence may be inherited (*12–16*). This was quite definitely demonstrated by crosses between strains A and C3H by Bittner (*17,18*) and Heston and Andervont (*19*). Andervont (*20*) showed the particular importance of hormonal stimulation in the origin of breast tumors in female hybrids from strains genetically predisposed to tumor development but lacking the milk agent. The influence of hormonal factors is not so evident in hybrids from strains with low susceptibility to the agent (*21*). This so-called inherited hormonal influence may be considered as a quantitative character. The level must vary from individual to individual and from strain to strain in a continuous distribution. In general, quantitative characters are controlled by a great number of genes and greatly modified by environment. Within any highly inbred substrain, the genetic background of hormone level should be the same, but variations among the individuals might occur due to environmental effects. These, in turn, could cause variations in tumor incidence.

Hormone level plays a most important role in maintaining homeostasis within the animal body. It seems probable that many environmental effects on tumor genesis are due to changes in hormone secretion or balance. Environmental factors, such as nutrition, temperature, activity (*22*), and so on, do affect tumor incidence. However, as far as this analysis is concerned, the effects of these specific factors cannot be detected. The linear relationship of number of litters produced and tumor incidence may be partially due to differences in total hormone stimulation among the individuals within a substrain, each successive pregnancy having added to this total.

Another relationship between tumor incidence and litters produced which should be mentioned is hypertrophy and lactation of the mammary glands associated with each pregnancy (*23*) and the tendency to formation of hypertrophic or precancerous nodules. These increase in frequency with successive pregnancies and may reflect differences in hormonal stimulation or may be related to the actual secretory functioning of the glands.

The nature of the mammary tumor milk-agent effect is much less clear than that of hormone influence on tumor genesis. At present, it is agreed that this agent may not be essential to tumor production (*21,24,25*), but it is a factor of stimulation to the mammary gland tissue which is largely transmitted through the milk but may in special cases come from either parent. It may be assumed to maintain a fairly even level within a strain or substrain and to vary between strains or substrains. Andervont and Dunn (*21*) reported less than 1 percent of tumor in their agent-free DBA females. Since the mice reported here have not been foster-nursed, milk agent is presumed to be present in all the DBA mice of this study, but its concentration may have varied between substrains and individuals within substrains.

Dmochowski (*24*) reported for two females of his (C57 × RIII)F_1

hybrids no tumors in litters produced before they developed mammary cancer, but tumors in litters produced after these females had shown breast tumors. Bittner's (*8*) observation indicated that individuals from the late litters showed more tumors. He interpreted this to mean that females had higher milk-agent concentration at late age. Our data, on a different strain, fail to corroborate his findings. In the present material, there may be an opposite relation between litter seriation and tumor incidence, with greater frequency in the offspring from early litters. Both sets of data, however, suggest that milk-agent concentration may vary at different stages in a female's life history.

This analysis has advanced understanding of mammary tumor genesis by disclosing significant effects of certain factors, including particularly environment common to litter mates, total litters produced by females, and minor genetic differences within one of the sublines. The relation of these variables to possible manner of action of the recognized tumorigenic agents has been discussed. Before closing this discussion it should be pointed out that the sum of all the effects tangible by this method accounts for less than one half of the total variance. The approximate contribution of each factor analyzed to the total variance of mammary tumor incidence in the five DBA substrains combined is:

Genetic factors	10%
Environment common to litter mates	19%
Litter seriation	4%
Number of litters produced	13%
Others	54%

In addition to disclosing factors responsible for variations in this body of data, these results suggest that in the design of future experiments studying mammary tumor genesis, the following points should be considered:

1) For the study of environmental effects, the basic stock must be in a static state with regard to tumor genesis. A genetically identical population is required, preferably with a long history of continuous brother × sister mating.
2) To compare individuals within a substrain or line, it is best, if possible, to split litter mates and to use animals either from the first two litters or from combined later litters.
3) Individuals should be classified by number of litters produced, or results should be corrected for this effect.

Summary

An analysis of mammary tumor incidence in strain DBA mice of the Roscoe B. Jackson Memorial Laboratory Inbred Nucleus stock has been carried out. It is based on the records of 517 animals of 6 different substrains. A description of the breeding history of each substrain has been given.

The breeding history and the results of the correlation analysis show different results for DBA/1(40)Jax than for the other substrains studied.

At the time of the investigation there still were significant dam-daughter and sibling correlations in this subline, suggesting genetic segregation and inheritance of difference in tumor incidence. In DBA/2HeJax these correlations were insignificant, and the variation in tumor incidence was caused entirely by environmental effects.

Littermate correlation coefficients of DBA/1(40) and DBA/2He were higher than dam-daughter correlation with regard to mammary tumor incidence. This suggests strong uterine and milk-agent effects.

Among the environmental factors, litter seriation possibly affects the tumor incidence slightly. That is, females from the first and second litters have higher tumor incidence than those from later litters.

Breeding females have higher tumor incidence than nonbreeders, and incidence increases with number of litters produced. Possibly this relationship was linear.

The contribution of each of the tangible factors to total variance of mammary tumor incidence has been discussed.

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Studies on Human Cancer Using Sponge-Matrix Tissue Culture. III. The Invasive Properties of a Carcinoma (Strain HeLa) as Influenced by Temperature Variations, by Conditioned Media, and in Contact with Rapidly Growing Chick Embryonic Tissue[1]

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Human carcinoma was found to invade normal human connective tissue *in vitro* in sponge-matrix culture (*1*). The normal tissue used was the outgrowth from explants of infant's foreskin; the human carcinoma was strain HeLa. Small inocula of HeLa cells invaded luxuriant cultures of the connective tissue and replaced the normal cells. In planning to use such a culture system experimentally, several factors must be considered. The pattern of invasion must remain unaltered in the presence of small variations in the temperature of incubation or of nutrient media conditioned either by the connective tissue or by the tumor cells. In addition, one wonders whether the phenomenon of invasion that has been observed in sponge matrix is really a parasitic one or largely the expression of differing rates of growth of tumor and normal cells. This report concerns experiments conducted to estimate the influence of these factors on the invasive properties of HeLa.

General Methods and Nutrient Materials

The methods for preparing sponge-matrix tissue cultures have been described (*2*). In the present study a prospective host tissue was planted in a combined matrix of cellulose sponge and plasma clot in a roller tube or in the flattened modification of the roller tube previously described (*3*). Either simultaneously or after the culture of normal tissue had been established a small inoculum of HeLa cells growing in a particle of Gelfoam was cemented to the host culture with the aid of a new plasma clot.

The liquid medium contained human serum, beef-embryo extract, and Hanks' balanced salt solution. An antibiotic content of 50 μg. per ml. of streptomycin and penicillin was present, as well as a concentration of 0.02 mg. per ml. of phenol red.

Cells and Tissues Used

Strain D.—This strain has been derived from cultures of a 4-day-old infant's foreskin that was originally explanted into sponge matrix on

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September 18, 1953. Subcultures were prepared by cutting the cultures with fine scissors into stringlike segments 0.5 to 1.0 mm. wide. These pieces were then placed on the free surface of a fresh slice of sponge and cemented in position with a plasma clot. Ten months after the original cultivation, this procedure was changed because more rapid growth was seen when the explants were sandwiched between the wall of the tube and the fresh slice of sponge before applying the plasma clot. Since isolation, the cells have been cultivated on media containing 30 to 40 percent human serum, with 1 to 5 percent beef-embryo extract. The *p*H of fresh medium was adjusted with 1.4 percent sodium bicarbonate to 7.6 to 7.8 before use. When the medium in the culture dropped to a *p*H of 7.1 it was replenished. At the time the experiments described here were conducted the strain was between 6 and 13 months old. It showed luxuriant growth 1 week after subculture, in contrast to the comparatively scant growth obtained from primary explants of foreskin after the same period. The histologic appearance of the subcultures, however, was indistinguishable from the outgrowth of primary explants; both produced a loose, fibrillary connective tissue.[5]

Strain HeLa.—HeLa, as used in this study, was cultivated in Gelfoam particle suspension (*1*,*2*). Data on the growth rate in this system have not been obtained. Subcultures were prepared about once a week. The medium used was the same as for strain D.

Embryonic tissues.—Various tissues of the chick embryo were used, including heart, frontal bone, tibia, liver, lung, brain, and intestine.

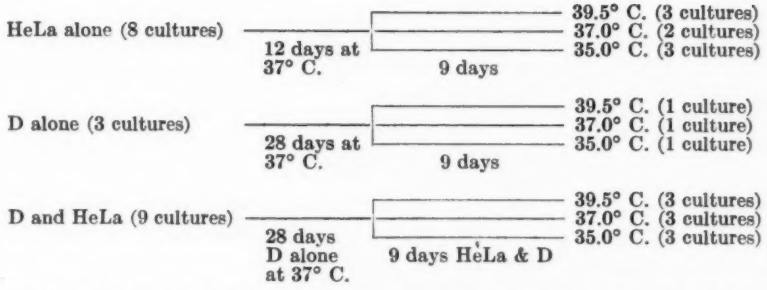
I. Temperature of Incubation

Small variations in temperature are found in many laboratory incubators, as well as in the patient. If differences of 2 or 3° C. were shown to produce marked fluctuations in the morphology of the aggregates of tumor cells, the normal cells, or the pattern of interaction of the two, studies on anti-tumor agents could be made with little reliability.

Twenty cultures were used in a comparison study at 35, 37, and 39.5° C. Some of the cultures were of strain D, some of HeLa, and some of the two cells combined. The distribution of cultures, as well as the history of the temperature of incubation, is shown in the chart, p. 1355. The incubators were equipped with recording thermometers which showed that there was never a deviation of more than 0.5° C. from the setting. The medium used in this series was composed of Hanks' balanced salt solution, human serum, and beef-embryo extract in the ratio of 10:5:1, with the addition of antibiotics and phenol red as indicated previously.

All the cultures were fixed after a 9-day period of incubation at the experimental temperatures. Zenker-formalin and alcoholic formalin (10% formalin in 95% alcohol) were used. The sponges were embedded

[5] Although these cells arose from normal infant's connective tissue, there has been reason recently to consider them as other than normal after more than a year in culture. During the second year of growth, strain D sometimes showed a quality suggestive of abnormality, such as large, bizarre, spindle-shaped cells which were occasionally seen in some areas of outgrowth. Further evidence of change away from the normal appeared 23 months after the original cultivation, when one culture abruptly changed and developed the morphologic characteristics of a highly malignant, anaplastic tumor (*4*).


HeLa alone (8 cultures)
12 days at 37° C.
9 days
39.5° C. (3 cultures)
37.0° C. (2 cultures)
35.0° C. (3 cultures)
D alone (3 cultures)
28 days at 37° C.
9 days
39.5° C. (1 culture)
37.0° C. (1 culture)
35.0° C. (1 culture)
D and HeLa (9 cultures)
28 days D alone at 37° C.
9 days HeLa & D
39.5° C. (3 cultures)
37.0° C. (3 cultures)
35.0° C. (3 cultures)

in paraffin, and serial sections were prepared on the plane of the sponge slice. Alternate slides were stained with hematoxylin and eosin. Selected slides were stained by Schiff's periodic-acid method, with Masson's trichrome stain, Mallory's phosphotungstic acid hematoxylin, and Regaud's iron hematoxylin. Between 20 and 50 slides were obtained from each culture.

Neither quantitative nor qualitative differences in the pattern of invasion could be found, nor were there any consistent differences within each of the two cell strains at the different temperatures.

II. Conditioned Media

The feeding schedules for cells *in vitro* vary considerably from one laboratory to another. In some a rigid feeding schedule is followed; in others the feeding intervals and volumes depend upon the demands of the cultures. The indices for a change in nutrient, in the latter case, may be a drop in *p*H as shown by a color change of the phenol red in the medium or microscopic evidence of depleted medium with rounding of cells and increased cytoplasmic granularity. With the dense cellular growth found in sponge-matrix cultures, only the index of *p*H change is of practical value. Two factors, the interval between feedings, and the volume of the feedings, may be varied in keeping the *p*H between 7.8 and 7.0. A practical plan is to give each culture a feeding estimated to be sufficient for 3 days. The estimate is often incorrect and in any series of cultures fed in this way, some must be fed earlier or later than anticipated. Before long, many of the cultures are completely out of phase. One culture in a group may thus have a longer exposure to the metabolic products of the culture or an exposure to higher concentrations of these products.

If small differences in exposure to the conditioned substances in the nutrient were shown to produce significant variations in the invasive pattern of strain HeLa and strain D, or in the quantity of invasion, such factors would be a major consideration in the design of experiments with anti-tumor agents. Furthermore, if a different pattern of invasion were observed when adapted medium from stock cultures was used as nutrient, an interesting avenue of study on the change in malignancy produced by nutritional contributions of the cells to the medium would be indicated.

Twelve cultures of strain D were prepared using a sponge slice in which a hole had been punched (*1*). A plasma clot cemented the inoculum of

connective tissue to the underside of the sponge near the edge of this "window." After 9 weeks, when the outgrowth of D cells covered the entire area of the window, small inocula of HeLa growing in Gelfoam particle suspension were placed in the punched-out area and cemented in position with a thin plasma clot. At the same time the cultures were divided into three equal groups, one receiving regular liquid medium, the second, HeLa-adapted medium, and the third, strain-D-adapted medium. All the cultures were kept in a roller drum and incubated at 35° C. They all received, initially, 1 ml. of the appropriate medium. The subsequent volume of the feedings was varied from day to day to maintain a *p*H change from 7.5 immediately after feeding to 7.1 to 6.9 twenty-four hours later. Each of the cultures received a thin patch on the 4th and the 8th days. They were all fixed after 18 days of cultivation with the 3 media. At this point the D strain was 13 months old.

Preparation of Media

Regular.—The regular medium consisted of the following components:

55 ml. Hanks' balanced salt solution
40 ml. human serum
1 ml. beef-embryo extract 50%
1 ml. antibiotic mixture
2 ml. heparinized chicken plasma
1 ml. chick-embryo extract 50%

These were added to a flask in the order given, mixed, and tubed out in 10 ml. aliquots within 15 minutes after the addition of the chick-embryo extract. The tubes were stoppered and kept at room temperature for 1 hour. During this period a soft clot formed. The tubes were then stored in the freezer. Upon thawing for use, a shrunken, floating clot containing particulate debris was easily removed, leaving a ruby-red, crystal-clear medium.

HeLa-conditioned.—Stock cultures of HeLa growing in Gelfoam particle suspension were fed with the regular liquid nutrient, 1 or 2 ml. per feeding. The medium was changed when the *p*H reached 7.1 to 6.9. The old medium was pooled in 10 to 15 ml. aliquots and stored in the freezer. Before use, the *p*H of the old medium was raised to 7.4 to 7.6 by the addition of small amounts of 1.4 percent $NaHCO_3$. The amount of bicarbonate required varied from one aliquot to another but was usually 5 percent, and never more than 10 percent, of the total volume. One percent glucose in distilled water was added in a volume equal to that of the bicarbonate. The medium was centrifuged for 15 minutes at 2,500 r.p.m., and the supernatant was used in feeding the cultures of HeLa and D combined.

D-conditioned.—Luxuriant stock cultures of strain D, growing in a combined matrix of cellulose sponge and plasma clot, were also fed with the regular liquid nutrient. The medium was replenished in the same way as for the HeLa cultures, and the same procedure was followed to obtain D-conditioned medium.

The pattern of invasion, as seen in the living cultures, was the same in each group. HeLa cells grew into the connective tissue both in the punched-out area and in the sponge around the window. As proliferation increased, the punched-out area became packed with tumor cells, and connective-tissue cells became increasingly hard to recognize. Packed tumor cells showed less cohesiveness than the interlacing cells of the connective tissue. Clumps of tumor cells broke off from the main mass as fluid moved over the cultures in the roller drum, and openings appeared within the tumor tissue occupying the punched-out area of the sponge. Thin plasma clots were applied to the cultures to reduce the erosion of the tumor mass. Liquefaction recurred in a short time and was most prominent in the cultures receiving HeLa-adapted medium.

The cultures were fixed with Zenker-formalin, Bouin's fixative, or 10 percent formalin. When the histologic sections of each group were compared, an obvious difference was seen in the total population of HeLa cells. In the cultures fed with regular nutrient, these appeared in great numbers as compact tumor tissue in the Gelfoam inoculum, as well as in the contiguous sponge and in the connective tissue. In cultures fed with D-adapted medium, HeLa cells were present in slightly smaller numbers. In the cultures fed with HeLa-adapted nutrient, the population of HeLa cells was considerably smaller both in the Gelfoam and in the neighboring connective tissue, although no evidence of degeneration or injury was seen in the tumor cells as compared with the two other groups. The D cells showed no changes from one group to the other.

Histologic sections from all three groups showed the area of the "window" to consist of a large zone of liquefaction, as well as the residue of the Gelfoam fragment containing HeLa cells. This fragment was eccentrically located, touching the sponge on one side of the circle as seen in figure 1. Connective tissue no longer was seen in the window itself, but condensed fibers were arranged around the circle, probably the collagenous residue of connective tissue previously occupying the open area. The tumor-cell outgrowth extended into the adjacent circle of connective tissue growing in the cellulose sponge. The pattern of outgrowth and invasion was the same in each group. In the Gelfoam residue of the inoculum, HeLa cells were arranged as a compact, densely cellular tissue supported by septa of incompletely digested Gelfoam (fig. 2). Tumor cells in dense masses formed the outgrowth immediately adjacent to the Gelfoam. Here all the connective tissue had disappeared. Slightly farther from the Gelfoam there was seen a compact tissue with orderly, short columns of tumor cells separated by parallel, and occasionally branching, eosinophilic fibers, the last residue of connective tissue (fig. 3). The zone blended into another in which HeLa cells were found among many spindle-shaped cells and condensed extracellular eosinophilic fibers. Peripheral to this was a connective tissue with a sparse population of fibroblasts and a loose, fine pattern of extracellular eosinophilic fibers in which HeLa cells either singly or in short rows could be seen (fig. 4). The eosinophilic connective-tissue fibers stained intensely red with the Schiff periodic-acid

reagent after diastase digestion. Variations in the three series were limited to the total number of HeLa cells present.

III. HeLa and Chick Embryonic Tissues

Finding that luxuriant cultures of slow-growing, normal, human connective tissue (strain D) were invaded by cells of the rapid-growing HeLa raised the following questions: Is the invasion of a slow-growing connective tissue by a rapidly growing tumor primarily the result of the different rates of growth? Would the invasive character of HeLa cells be manifest if they were cultivated with embryonic host tissues which themselves grow rapidly in tissue culture? To find the answer HeLa was combined with several tissues of the chick embryo in sponge-matrix culture. Table 1 shows the embryonic tissues used, the time at which strain HeLa was added to the cultures, the composition of the nutrient, the length of time of cultivation, and other details. All the cultures were incubated at 35 to 37.5° C. and kept in a roller drum.

Chick-embryo heart.—Explants of chick-embryo heart in sponge matrix gave rise to a luxuriant connective tissue which contained many capillaries.[6] Endothelium-lined spaces were often so numerous, especially around the explant, as to present an angiomatous pattern made up of contiguous anastomosing cavernous spaces (*5*). In both series of cultures the HeLa cells were seen, both in the living culture and on histologic examination, to be invading the margins of the connective-tissue outgrowth from the chick-embryo heart. Figure 5 shows a field from a Wilder reticulum preparation of a culture in the first series. Tumor cells are seen migrating into the loose mesh of reticular fibers between the spindle-shaped cells. In the lower half of the figure several capillaries are seen.

Chick-embryo bone.—Growth of a connective tissue with many spindle-shaped cells was obtained from both the tibia and the frontal bone, though the growth was much more luxuriant from the frontal-bone explants. Growth from the HeLa inoculum was also extensive, and a very close intermingling of the tumor cells and fibroblasts from the bone was seen, especially in the frontal-bone series. Histologic examination showed extensive liquefaction, particularly in the tibia series. The outgrowth from both kinds of bone consisted in part of a membrane of connective tissue which formed an almost complete coating about the trabeculae of the explant. Connective tissue extended out into the sponge interstices as well. Islands of HeLa cells were found throughout the sponge. Tumor cells were also seen invading connective tissue in many areas, including the membrane of connective tissue that coated the bone explant. In a few areas HeLa cells infiltrated this membrane deeply and were in contact with the trabeculae of bone (fig. 6).

Chick-embryo liver.—When embryonic liver and HeLa cells were combined, plasma-clot liquefaction took place so rapidly that a suitable

[6] A similar pattern of growth has been seen from explants of adult human heart, with an extensive lattice of branching capillaries growing into the sponge interstices in the course of a month. The tissue was from an auricular appendage, removed as part of an operation on the mitral valve.

TABLE 1.—*Data on HeLa cells combined with chick embryonic tissues*

Chick embryonic tissue	When HeLa in Gelfoam was added	Number of cultures	Nutrient	Length of time of cultivation after addition of HeLa (*days*)
Heart, 1 explant 1.5 mm. in diameter, from 11-day-old embryo.	At the same time.....	2	Hanks' B.S.S.; Human serum; Beef E.E. 10:5:1	7
Heart, 2 or 3 explants, from 10-day-old embryo.	Eight days later and cemented with plasma clot.	2	Hanks' B.S.S.; Horse serum; Chick E.E. 2:2:1	13
Tibia, 1 explant 1 to 2 mm. long, from 14-day-old embryo.	When clot cementing tissue was firm, HeLa was cemented in position with fresh clot.	3	Regular liquid nutrient;* occasional patching.	36
Frontal bone, 2 or 3 explants each 1 mm. in diameter, from 14-day-old embryo.	At the same time.....	3	Regular liquid nutrient; occasional patching.	22
Liver, finely divided pieces totaling 2 mm. in diameter, from 14-day-old embryo.	At the same time.....	3	Regular liquid nutrient; frequent patching.	14
Brain, 1 explant 2 mm. in diameter, from 14-day-old embryo.	At the same time.....	3	Regular liquid nutrient; frequent patching.	14
Lung, 2 or 3 explants, from 14-day-old embryo.	At the same time.....	3	Regular liquid nutrient; frequent patching.	11
Lung, 2 or 3 explants, from 15-day-old embryo.	Six days later.....	4	Regular liquid nutrient; frequent patching.	10
Intestine, fine mince of piece 3–4 mm. long, from 15-day-old embryo.	Three days later.....	4	Regular liquid nutrient; frequent patching.	8—2 cultures; 11—2 cultures
Intestine, fine mince of 3–4 mm. piece, from 15-day-old embryo.	Six days later.....	4	Regular liquid nutrient; frequent patching.	10

*See section on Conditioned Media.

physical substrate for the migration of embryonic cells or tumor cells could not be maintained, and the explants of each remained discrete and circumscribed. On histologic examination the cultures showed almost no plasma clot in the sponge interstices, although growth of HeLa cells around the Gelfoam inoculum was extensive. The liver was made up of cellular masses such as large groups of vacuolated, hepatic, epithelial cells on the surfaces of the explants in some areas, and zones with densely packed spindle-shaped cells. Areas with a lattice work of endothelium-like cells lining open spaces, and groups of epithelial cells covered by flattened endothelium-like cells and separated by open sinusoid spaces were also seen, as previously described (*5*). In the occasional areas where HeLa cells and liver tissue were found together, the tumor cells were arranged on the surfaces of the embryonic tissue in an irregular layer, and were also seen as small groups of cells within the substance of the embryonic liver tissue (fig. 7).

Chick-embryo brain.—As in the case of liver and HeLa, the cultures of embryonic brain and HeLa showed, on histologic examination, that areas of contact between the two were infrequent. Here, too, plasma-clot liquefaction was complete, so that no suitable physical matrix was present for the support of migrating tumor cells. However, in a few areas, tumor cells were found infiltrating the brain tissue, as seen in figure 8. The brain presented a varied picture, with necrosis in some areas and many cells with small oval nuclei in other areas. Some of the cells were bipolar with scant cytoplasm. Others showed stellate or polyhedral contours with eccentrically located nuclei and abundant eosinophilic cytoplasm. In some areas, particularly at the surfaces of the growth, but also around small lumens in the deeper portions of the explant, the cells were more hyperchromatic and were arranged in regular palisading fashion (fig. 8).

Chick-embryo intestine.—In the living cultures, outgrowth from the explants of embryonic intestine consisted of short-lived sheets of epithelium which retracted to the explant as the plasma clot liquefied. The explants of intestine developed smooth surfaces lined by a regular columnar epithelium after 2 or 3 days. Clumps of HeLa cells were in direct contact with the chick tissue in many areas, but no definite invasion of the chick tissue was seen in the living cultures. On histologic section it was seen that the fragments of chick intestine had undergone a histologic reorganization, as described by Fischer many years ago (*6*). The surfaces of most of the fragments were completely covered by a columnar epithelium. The lamina propria consisted of a narrow zone of loose mesenchyme-like connective tissue, and the greatest part of the tissue forming the center was composed of dense connective tissue and smooth muscle.

Invasion by HeLa through the intact epithelial surface of such intestinal "organisms" was absent, and if "invasion" had taken place where normal embryonic intestinal epithelium and carcinoma cells met, it was invasion by the normal cells. Chick intestinal epithelium was sometimes found to be spreading on the surfaces of densely packed HeLa in Gelfoam explants and occasionally migrated into the midst of the HeLa cell

population of a Gelfoam fragment (figs. 9 and 10). Only in occasional areas, where the continuity of the intestinal epithelium was broken and the underlying connective tissue was sparsely cellular or necrotic, did HeLa show signs of invading the stroma of an intestinal organism (fig. 11). The findings in a few fields may have represented the end stage of this process. Here fragments of chick-embryo intestine appeared to have fared badly and consisted of a few pale-staining surviving connective-tissue cells in a dense, eosinophilic, collagenous matrix. Adherent HeLa cells appeared to be using this residue as a physical support.

Chick-embryo lung.—When embryonic lung tissue and HeLa were planted at the same time, liquefaction of plasma clot was very marked, and freshly applied patches of clot were usually completely liquefied overnight. Migration of cells from the explants was limited by the speed of liquefaction, and areas of contact between HeLa cells and lung tissue were not recognized with certainty in the living culture. On histologic study the lung fragments showed some characteristics of "organ culture," and appeared as a fairly circumscribed, densely cellular embryonic glandular tissue. The surface of the lung tissue was partially covered by polygonal cells, which were the largest of the embryonic cells. These extended for short distances away from the surface of the explant in some areas, obtaining their physical support from the cellulose sponge trabeculae. Elsewhere they made a narrow layer of stratified cells on the surface of the lung explants, without forming distinct histologic structures. The HeLa inocula in Gelfoam were, for the most part, not in contact with the lung fragments. In a culture where the two tissues were touching, several groups of actively growing tumor cells were seen within the lung tissue, and there was an intimate mingling of the large, polyhedral embryonic cells and the HeLa cells (fig. 12).

In the second series, in which the lung was explanted 6 days before the addition of HeLa, progressive growth from the lung fragments was luxuriant in the living cultures and consisted of groups of cells, both spindle-shaped and polyhedral, which formed an extensive branching pattern as they bridged many sponge interstices. Clot liquefaction occurred, but many of the newly formed structures that spanned the interstices of the sponge persisted, maintaining an anchorage on the cellulose trabeculae. Large, multilocular, cystlike structures appeared in the bridges of tissue after a few days, persisted, and became more numerous during the course of the experiment. When HeLa was added, the outgrowth of tumor cells was abundant and appeared to be in contact with embryonic cells in many areas. In the histologic sections the sites of implantation of the embryonic tissue were recognized by the presence of small pieces of cartilage. The outgrowth from these areas consisted of an irregular, polypoid lung tissue that extended into the interstices of the sponge and was covered by a single-celled layer of low cuboidal or squamous epithelium (fig. 13). Most of the substance of the "polyps" consisted of sparsely cellular, loose, mesenchyme-like connective tissue. (Spherical structures of this composition were interpreted as cystlike

structures in the living culture.) The mesenchyme was made up of small, spindle-shaped cells and histiocytes, and occasional spaces were lined by a flattened cell layer resembling endothelium. In addition, there were occasional, small aggregates of epithelial cells in cord, or glandular, arrangements. Where masses of HeLa were in contact with the epithelium-like surface of the polypoid outgrowth, or with the residue of the explant itself, only an occasional site of tumor infiltration into the lung tissue could be found (fig. 14). Peripheral to the "polyps" were strands of polyhedral macrophage-like cells that extended for some distance into the sponge interstices (fig. 15). In the absence of plasma clot or large connective-tissue masses, these cells were supported by their adherence to the cellulose sponge. They resembled the cells seen on the surfaces of the lung explants in the first series. These most peripheral cells of the outgrowth showed very intimate mixing with cells of strain HeLa. In some places the mixing was in an apparently random fashion, in others the two formed small structures in which the tumor cells were surrounded by a single layer of cells from the lung (fig. 16).

Discussion

The experiments described here were carried out as prerequisites to the evaluation of pharmacologic agents on test systems of sponge-matrix tissue culture in which tumor cells invade normal tissues. We studied the influence on the invasive capacity of HeLa cells of small variations in temperature of incubation, and of media conditioned either by the host or by the tumor cells. For these studies HeLa cells were combined with a strain of normal human fibroblasts (strain D). In addition, experiments were conducted in which HeLa cells were added to cultures of chick embryonic tissues so that these two kinds of rapidly growing tissue might be observed as they made contact with each other.

Small *variations in temperature* were found to influence neither the pattern of invasion nor the total amount of growth as measured by the semiquantitative method of examination of serial histologic sections.

When *conditioned media* were substituted for the regular nutrient, an effect was seen with HeLa-conditioned medium that was not seen with the other media. The total mass of HeLa cells was markedly diminished in the Gelfoam inoculum and within the connective tissue, although the histologic invasive pattern was not affected. It is unlikely that this kind of variation would be a serious factor in evaluating the effects of pharmacologic agents, since the method of preparing the conditioned medium was extreme. For example, an increase of a day in the feeding interval would not alter the medium to the same extent as in our experiment. The influence of alterations in the medium due to conditioning can be reduced if feedings are planned in which the nutrient is replaced every day and the volume of medium is varied.

The small population of HeLa cells, along with the histologically normal connective-tissue cells, found in the cultures fed on HeLa-adapted medium is of interest in itself. The factors involved in this effect will require

further study; there are two which we consider particularly accessible to experimentation. First is the possibility that a medium that can support the normal host tissue adequately may fail to provide the tumor cells in the same culture with the substances needed for their growth. The tumor tissue may not be able to make up its dietary deficiency completely by invading, destroying, and utilizing the nearby normal tissue. The parasitic properties of the tumor cells by which they exist on normal tissues may require a humoral nutritive supplement. If this, in some circumstances, is actually true for tumor and host tissues within the body, chemical agents for the treatment of cancer may be more readily developed. Secondly, liquefaction of the plasma clot may alone account for the differences observed. The small population of HeLa cells found in the presence of HeLa-adapted medium may be the result of the increased liquefaction of the plasma clot found in this group and the accompanying loss of groups of tumor cells into the fluid medium. The significance of clot liquefaction in the development of the eventual histologic picture of a tissue seen in sections of sponge-matrix cultures is very great, and it must be defined further in studies with clots of varying physical consistency and in the presence of trypsin inhibitors. Macrophages are the only cells which cling avidly to the cellulose sponge long after complete liquefaction of the plasma clot has occurred. Other cells require the physical support provided by the fibrin meshwork of a plasma clot within the cellulose interstices, or a connective-tissue bed that has replaced the plasma clot within the sponge. Connective tissues will remain stabilized after the plasma clot is replaced, this stability being obtained by means of bundles of collagen fibers that span the sponge interstices; the tissues are anchored in many places as they loop around the cellulose trabeculae.

The data obtained in the case of *chick embryonic tissue and HeLa in combination* are fragmentary, but they show that the pattern of interaction may be extremely varied. The factors involved here are complex, especially for epithelial organs. In the combinations of embryonic lung and embryonic intestine with HeLa, cells of the normal tissue were found to "invade" the explant of tumor cells in Gelfoam particles. These observations raise the possibility that normal host cells in the body may in certain circumstances "invade" discrete nodules of potentially malignant cells and mechanically disperse the tumor cells, establishing additional new foci for growth of tumor cells and accelerating the spread of the malignant process.

Combinations of HeLa and embryonic epithelial tissues are especially difficult to work with, since they liquefy plasma clot at a rate far above the capacity of the embryonic connective tissue to replace the plasma clot with new connective tissue. Under these conditions physical pathways to support the migration of cells are absent, and only the random attachment of a floating tumor cell to an island of embryonic tissue in the sponge can take place. Furthermore, in pharmacologic studies the use of a complex host tissue with glandular components would present an additional complication, since one might not be studying the direct

influence of the chemical agent on the mechanism of invasiveness, but rather an indirect effect as the chemical acts on functional glandular tissue in the explant.

On the other hand, the method of combining HeLa with a host tissue consisting of an established culture of connective tissue having no epithelial components lends itself readily to the recognition and qualitative evaluation of changes in the tumor such as maturation, differentiation, or cell degeneration, as well as responses on the part of the host tissue which tend to limit the growth of the tumor cells.

One finding common to all of the invasion experiments described here introduces another problem. In no instance where HeLa invades a normal tissue, whether strain D or chick embryo, can the remains of the host cells be found. It is almost as if normal connective-tissue cells, displaced by tumor cells, promptly disappear rather than leave a residue of visible products of degeneration. The morphologic fate of the normal cells can only be guessed at with static histologic techniques, but perhaps the process can be studied profitably with the use of time-lapse cinematography.

It is highly desirable to develop quantitative methods for measuring many of the aspects of cellular interaction in tissue culture. Refinements of method are under study with which the over-all rate of the invasion can be determined.

Summary

Some aspects of the physiology of the invasion of normal tissues by tumor cells were studied *in vitro* by combining a human carcinoma (strain HeLa) with several normal host tissues. The effects of temperature variations and of conditioned media were examined in cultures of HeLa and a strain of normal human connective-tissue cells (strain D). Also studied was the interaction of HeLa and rapidly growing chick embryonic tissues.

1) Variations in temperature of incubation from 35 to 39.5° C. did not change the pattern of invasion.

2) Conditioned media, taken from stock cultures of either HeLa cells or strain D cells, and substituted for the regular liquid nutrient in cultures of the two cells combined, did not influence the histologic pattern of invasion. The total growth of HeLa, however, was markedly diminished in the presence of HeLa-conditioned medium.

3) Rapidly growing cultures of several chick embryonic tissues were inoculated with HeLa cells. Extremely varied patterns of interaction developed. The mesenchyme-like embryonic connective-tissue outgrowths from heart and bone tissue were extensively infiltrated by HeLa cells. The HeLa cells invaded embryonic liver and brain tissue, but the extent of the invasion was difficult to determine and was confused by the factor of clot liquefaction. The interpretation of the pattern of interaction in combinations of pulmonary or intestinal embryonic tissue growing with tumor was even more complicated; other factors were involved here in addition to the difficulties introduced by marked lique-

faction of the plasma clot. An intact layer of intestinal columnar epithelium and of some pulmonary epithelium appeared to be unaffected by the invasive capacity that HeLa cells exhibit when combined with connective tissue. Moreover, cells of the embryonic intestine and lung were found to "invade" explants of HeLa cells in Gelfoam particle suspension.

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PLATE 118

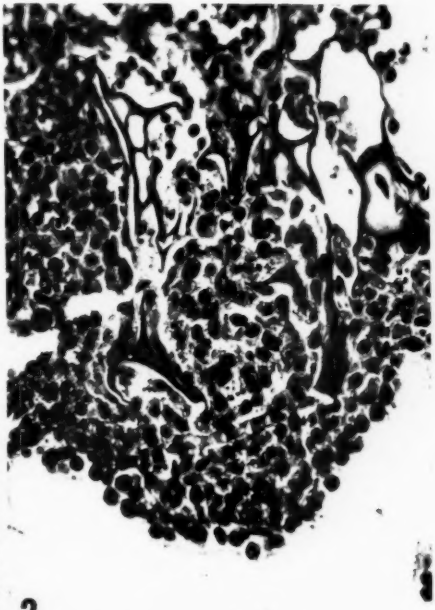
Histologic sections showing the pattern of growth and invasion of strain HeLa cells into human connective tissue (strain D)

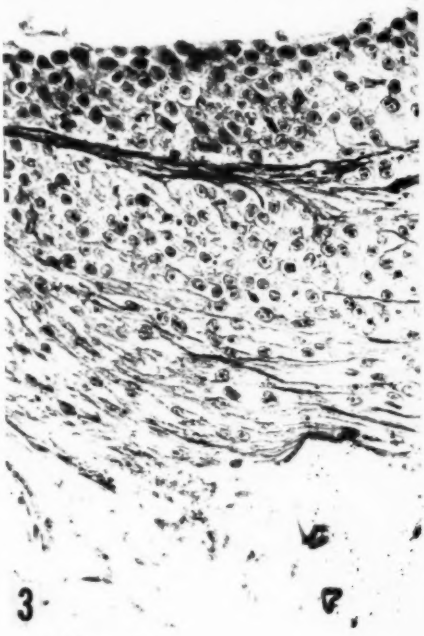
FIGURE 1.—The edge of the punched-out opening in the sponge is seen with low magnification. HeLa cells have grown out from the Gelfoam inoculum (*upper left corner*) and have invaded and replaced most of the connective tissue that previously occupied this entire area. The clear zone in the left half of the field is the result of desquamation of poorly cohesive tumor cells. The tumor is many cell layers deep around the edge of the window and is seen infiltrating the adjacent connective tissue. Hematoxylin and eosin. × 73

FIGURE 2.—Under higher magnification, the area of the inoculum shown in figure 1 is seen. The residue of Gelfoam provides a supporting matrix for the HeLa cells. Hematoxylin and eosin. × 250

FIGURE 3.—A view of the edge of the punched-out area of figure 1 shows HeLa many cell layers deep. The prominent, condensed fibers are probably a residue of the connective tissue that has been replaced. Periodic acid-Schiff with diastase. × 250

FIGURE 4.—HeLa cells and connective-tissue cells are intermingled in the invading margin of HeLa cells. Hematoxylin and eosin. × 250





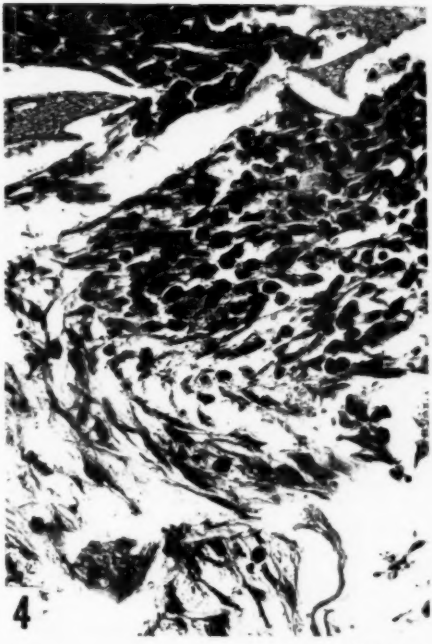


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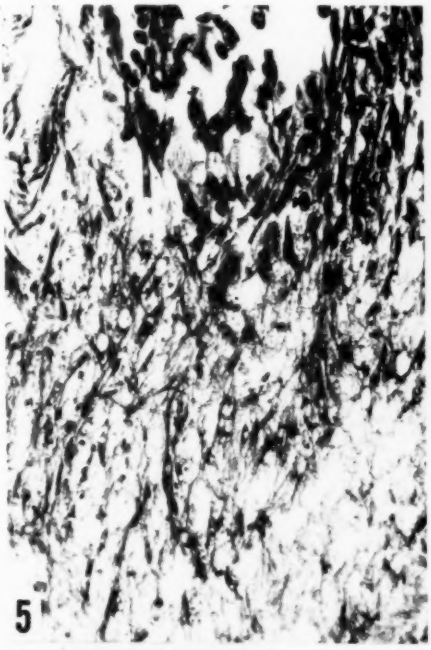
PLATE 119

FIGURE 5.—Histologic section of a culture of cardiac tissue of the chick embryo combined with HeLa. Tumor cells in the *upper half* of the field are invading connective-tissue outgrowth from the heart. Capillaries are seen in the *lower half* of the figure. Wilder's reticulum. × 250

FIGURE 6.—Culture of osseous tissue of the chick embryo being invaded by tumor cells which are in contact with bone trabeculae. Periodic acid-Schiff. × 250

FIGURE 7.—Culture of tissue of the chick embryonic liver invaded by HeLa cells. Large, hyperchromatic tumor cells are seen in the midst of many smaller, vacuolated liver epithelial cells. Hematoxylin and eosin. × 250

FIGURE 8.—Culture of tissue of the chick embryonic brain combined with strain HeLa. Tumor cells are seen in the *upper half* of the field among cells of the embryonic brain. Hematoxylin and eosin. × 250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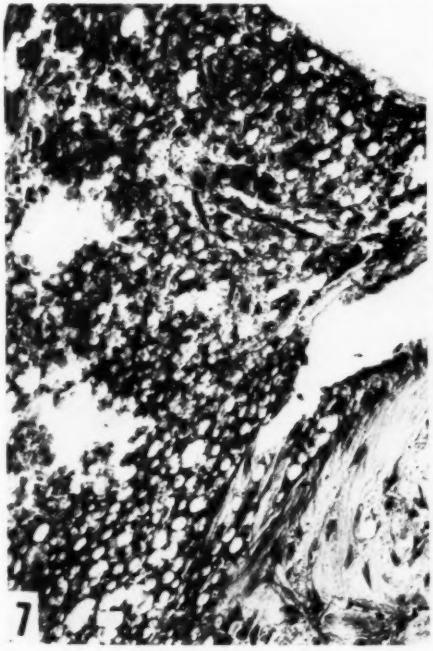
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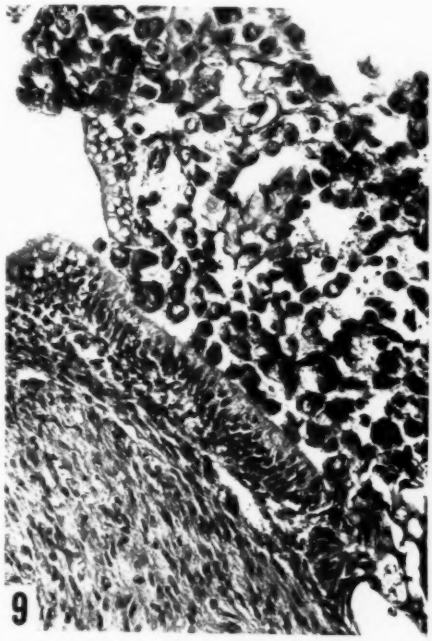
PLATE 120

FIGURE 9.—Explant of HeLa in Gelfoam and embryonic intestinal epithelium of the chick in close contact. The tumor cells have not disturbed the integrity of the epithelial surface lining of the "intestinal organism." In the *upper* part of the figure, a layer of embryonic columnar epithelium has spread out to cover part of the surface of the Gelfoam explant. Hematoxylin and eosin. × 250

FIGURE 10.—An area of the Gelfoam explant of figure 9 in which a cyst lined by embryonic intestinal epithelium of the chick is seen deep in the HeLa-Gelfoam inoculum. Hematoxylin and eosin. × 250

FIGURE 11.—An area in which HeLa cells are in contact with degenerating, sparsely cellular connective tissue of intestine from the chick. The intestinal epithelium on each side of the area is extremely vacuolated. The HeLa cells and segments of normal-appearing intestinal epithelium show a very markedly positive reaction with the Schiff periodic-acid method. × 250

FIGURE 12.—HeLa and embryonic lung of the chick in contact. An intimate mixing of HeLa cells and smaller, macrophage-like cells of the lung is seen. Regaud's iron hematoxylin. × 120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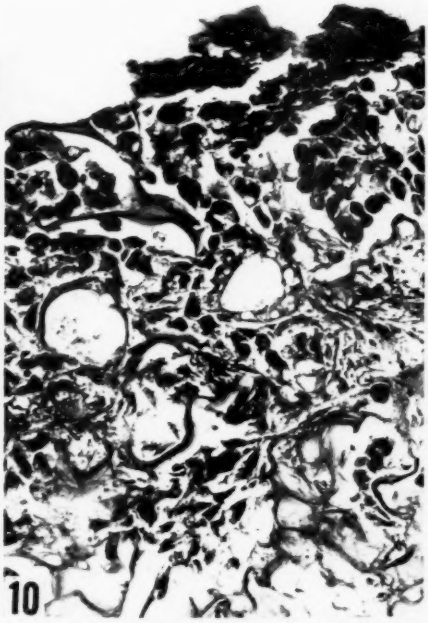
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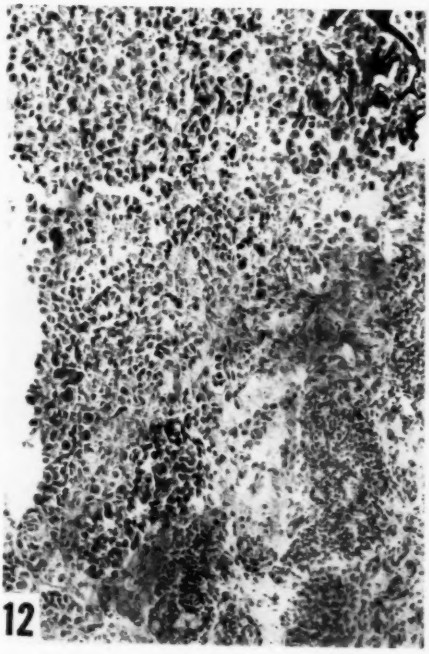
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PLATE 121

FIGURE 13.—Polypoid outgrowth of embryonic lung of the chick extending into the interstices of the cellulose sponge. Hematoxylin and eosin. × 250

FIGURE 14.—Large mass of HeLa cells (*left*) is in direct contact with the surface of a polyp. No invasion by either tissue is seen. Periodic acid-Schiff. × 250

FIGURE 15.—The most peripheral outgrowth of the chick-embryo lung consisting of polyhedral cells with small nuclei and nucleoli. Hyperchromatic, large HeLa cells mingle with the cells of the lung in no obvious pattern. Hematoxylin and eosin. × 250

FIGURE 16.—Peripheral outgrowth of chick-embryo lung and HeLa cells. An organized aggregate is seen in which the tumor cells are surrounded by a single layer of cells from the lung. Regaud's iron hematoxylin. × 250

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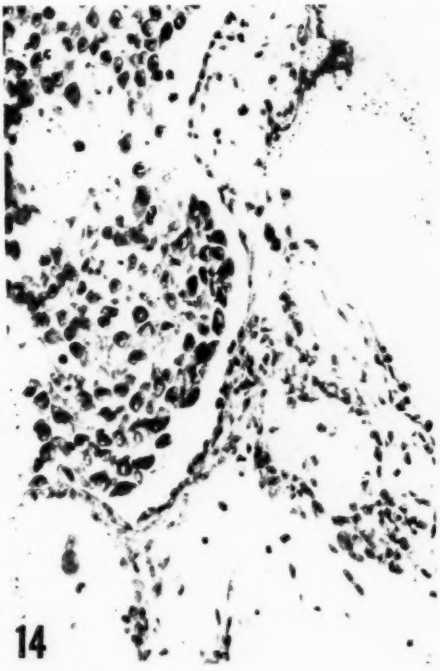
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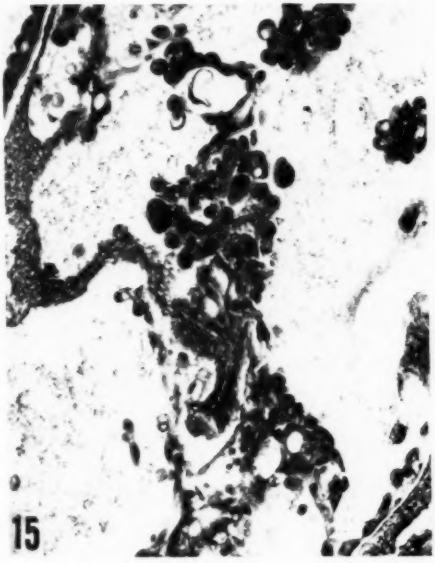
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16

The Growth of Cells on a Transparent Gel of Reconstituted Rat-Tail Collagen[1,2]

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With the knowledge that collagen is a universal component of connective tissues, serving as a kind of solid substratum and support for cells in the organism, an effort has been made to reduplicate some aspects of this natural system in tissue cultures. The ready availability of collagen and its stability lend themselves to such a study. Early work, done chiefly by Huzella and associates between 1932 and 1944 (*1–6*), was directed toward studying the behavior of cells on fibrous collagen, either natural, or reconstituted by salt precipitation from acetic-acid solutions. The cells attached themselves and grew along these fibers in a manner similar to their growth on other kinds of animal, vegetable, or synthetic fibers (*7,8*). With the finding (to be reported here) that collagen can be reconstituted from solution as a transparent gel or as a cellophane-like sheet, a new pathway has been opened for studying the behavior of cells on collagen—the cells no longer being restricted to growth around an individual fiber. An additional incentive for using collagen in tissue cultures stems from our unsatisfactory experience with the cultivation of some types of cells in continuous cultures on glass (which is reported here) or in the highly digestible fibrin clot (*9–11*) when not protected against lysis by heated serum (*12*) or by soybean trypsin inhibitor (*13,14*). Growth of cells on other kinds of gels and membranes have also proved generally unsatisfactory for continuous cultures (*8*), with the possible exception of cultures grown in a three-dimensional gelatin sponge matrix (*15*).

The earliest known work on collagen preparations dates back to 1900, when Zaccharíades (*16*) made extracts of rat-tail tendon with dilute mineral acids. Considerably later, Nageotte (*17–19*) discovered that by adding salt to acid extracts of rat-tail tendon, he could precipitate collagen in fibrous form. Similar findings on fish collagen (ichthyocol) were made in 1937 by Fauré-Frémiet and Garrault (*20*). Despite the important

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finding of Nageotte and Guyon (*21*), Fauré-Frémiet (*22,23*), and Leplat (*24,25*) that collagen could also be reconstituted in the form of a transparent gel, the fibrous form attracted much more attention. In 1942, Schmitt *et al.* (*26*) showed by electron micrographs that reconstituted collagen fibrils have the banded structure characteristic of native collagen. This initiated intensive studies on the fine structure of collagen, using as a tool the reconstitution of collagen fibers from solution under various experimental conditions (*27–43*). The work between 1900 and 1953 has been reviewed more extensively by Fitton Jackson and Randall (*40*).

In addition to this work on collagen fibrogenesis and structure, collagen presents other interesting properties. For example, acetic-acid solutions, when evaporated to dryness, leave a microscopically transparent sheet like cellophane, only more brittle.[5] Sheets of this sort show the wide-angle X–ray diffraction pattern typical of collagen, while much thinner membranes prepared by drying dilute acetic-acid solutions on collodion films show no fibrils of any kind under the electron microscope.[5] When more concentrated collagen solutions are dried into thin films on formvar (*32*) or on collodion (*34*), electron micrographs show fine, structureless filaments varying in width from 30 to 400 Å, and in length up to 400 mμ. These ultramicroscopic studies, plus the observation that low collagen concentrations can produce highly viscous solutions with flow birefringence (*32*), and experiments on the scattering of light by collagen solutions (*37*) indicate that acetic-acid solutions of collagen contain long, threadlike macromolecules.

In working with various techniques, an interesting transformation was found to occur in solutions of rat collagen when dialyzed against distilled water. After about 2 days, the viscosity begins to rise rapidly, with complete gelation in another 24 to 48 hours—the gel being clear and firm. One might conclude that the filaments have arranged themselves into a molecular lattice. Microscopically visible stress fibers sometimes appear in a gel slice while serving as a substratum for growing cells in tissue culture. This may be evidence of the filamentous structure. Mild warmth (37° C.) causes rapid syneresis of the collagen gel. When raised to the boiling point, the dispersed collagen quickly contracts down to a tiny white thread which floats around in the syneresed fluid. Cooling does not lead to re-gelation as it would in gelatin. These findings are essentially in agreement with those of Nageotte (*21*). A slice of the dialyzed collagen gel, like the acetic-acid solution, dries down to a transparent cellophane-like sheet (fig. 1). Later studies indicated that wrinkling could be avoided if the dialyzed gel were dried over a hole cut in a taut sheet of cellophane rather than directly over a metal hoop. Gelation of an acetic-acid solution of collagen can also be brought about by exposure to ammonia vapor, but this produces a fine granularity within the gel.[6] The submicroscopic structure of collagen gels or of gels dried down to cellophane-like sheets is being explored.

[5] Dr. Jerome Gross, personal communication.
[6] See Addendum.

Procedure

Isolation of Rat-Tail Tendons and Extraction with Acetic Acid

The rat-tail tendons were obtained in a manner similar to that used by Watson *et al.* (*44*). Beginning at the tip, the tail was successively fractured into small pieces by means of two Kelly clamps. Each piece, in turn, with its attached tendon strands was pulled free from the remainder of the tail before making the next fracture. Pied rats were used, and freshly obtained tails were immediately stored in the deep freeze. The skin was not removed as in Watson's procedure, but the tail was soaked in 95 percent alcohol sponges for 15 minutes prior to fracturing. The long, silvery tendon strands were cut free from each new tail segment as it was broken off and allowed to drop into a petri dish containing distilled water. Strands adherent to the scissors were freed with forceps. With two pairs of fine curved forceps, the pooled tendon strands from 1 tail were teased apart into finer filaments and then removed en masse to a sterile 250 ml. centrifuge bottle containing 150 ml. of 1 : 1,000 acetic-acid solution. The bottle was sealed and stored for 48 hours in the refrigerator, during which time the tendon strands swelled, usually until the total volume became occupied by a translucent jelly-like mass. Centrifugation for 2 hours at 2,300 r.p.m. brought down the remaining solid residue. About 40 ml. of viscid fluid, either clear or finely flocculent, were removed at this stage and pipetted into a large test tube labeled S1. About 30 ml. of sterile 1 : 1,000 acetic acid were added back to the residue and thoroughly mixed with it by shaking the bottle. This new mixture was usually cloudy with fine floccules, even if the fluid just removed was clear, indicating that the centrifugation itself had helped to break up the fragile swollen tendons. The mixture was stored for 24 hours in the refrigerator, and centrifuged again for 2 hours at 2,300 r.p.m. This time, between 40 and 80 ml. of viscid fluid could be removed by pipette and labeled S2. The residue was shaken without adding any more acetic acid and stored for another 24 hours in the refrigerator. After 2 hours' centrifugation, from 30 to 70 ml. of viscid fluid were removed and labeled S3. In extractions of 10 different rat tails, the total of S1, S2, and S3, averaged 132 ml., but varied between 60 and 174 ml. Cloudy or flocculent supernatant fluids were clarified by ultracentrifugation at 25,000 r.p.m. for 30 minutes. The crude-collagen content of the various supernatant fluids was determined by dialyzing 10 ml. aliquots against distilled water to eliminate the acetic acid, followed by lyophilization and weighing. The yields averaged 0.17 gm. per 100 ml. of fluid supernatant, and varied between 0.12 and 0.23 gm. percent. There was no consistent decrease or increase in collagen yields in going from S1 to S3. The supernatant fluids could be stored without deterioration for over 7 months in the refrigerator, and could also be stored in the deep freeze.

Reconstitution of Collagen as a Gel by Exposure to Ammonia Vapor

Exposure of the acetic acid-collagen solution to ammonia vapor rapidly converted it into a slightly hazy, soft gel which microscopically showed

very fine granules.[7] For experiments with cultured cells, 0.5 ml. of acetic acid-collagen solution was added to roller tubes. These were plugged with cotton moistened with 28 percent ammonia and laid on their sides at a slight angle for about an hour. The resultant gel (resembling an agar slant) was washed for at least 24 hours with several changes of sterile distilled water, balanced salt solution (Gey), and equilibrated with the medium to be used. The preparation was soft enough to allow cells to migrate down into it, but the penetrating cells were always severely retracted.

Reconstitution of Collagen as a Gel by Dialysis against Distilled Water

About 30 ml. of acetic acid-collagen solution were placed in an autoclaved cellophane dialysis bag (Will Corporation), whose dimensions when filled were approximately those of a sausage 3 inches long and 1½ inches in diameter. After 2 days of dialysis in the refrigerator against distilled water, the viscosity of the contents started to increase, as shown by the retarded movement of trapped air bubbles when the bag was inverted. In another day or two, the bubbles remained motionless on inverting the bag, indicating gelation. The bag contents sometimes developed a faint opalescence at this stage. After a total of 6 to 11 days of dialysis, the bag was taken out of the water, blotted with a dry sterile-gauze sponge, and then held over a glass container of sterile distilled water in which was submerged a sterilized modified egg slicer with fine steel wires spaced ⅛ inch apart. With a pair of sterilized scissors, the dependent tip of the bag was snipped off. Squeezing the bag slightly at the uppermost end was enough to start the jelled contents to slide slowly out of the bag and settle in the hollow of the submerged slicer. The wires were brought down over the sausage-shaped gel, slicing it into about 20 discs which were scooped out with a stainless-steel teaspoon and divided equally between 2 petri dishes of sterile distilled water.[8] The discs were separated with a thin, narrow spatula. An individual disc was lifted out of the petri dish with the spoon and placed on a fluted spatula. The tip of the fluted spatula was inserted into the mouth of a roller tube held at an angle. Shaking the spatula slightly caused the gel disc to slide down into the roller tube, which was quickly brought to the horizontal when the disc was about an inch from the bottom. Adjustments in position were made by tipping the tube one way or the other, and folded-over edges were gently corrected with the thin spatula. The tube was laid on its side with the disc down for several minutes, then rotated in place to bring the disc on top, in which position it was left for at least 15 minutes. If adherence of the gel was satisfactory, the tube was carefully tipped, allowing the water to drain off. The mouth of the tube was flamed, and the tube was placed vertically upside down on sterile-gauze sponges in a can. Here it was left for several hours to allow the gel to adhere more firmly during partial

[7] See Addendum.

[8] In early experiments, the gel was sliced with a scalpel rather than an egg slicer, but this proved to be a very difficult and variable technique.

dehydration. If the gel disc failed to adhere at the outset, it was poured from tube to tube until one was found to which it did adhere. When these manipulations were carried out at room temperature little difficulty with adherence was encountered. However, even a slight elevation of temperature prevented good adherence. Gel slices can be stored in 95 percent alcohol without loss of transparency, and preliminary data indicate that cells can be cultivated on such collagen following alcohol storage.

Equilibration of Dialyzed Collagen Gel with Culture Medium

Inspection of the disc after adherence to the roller tube showed it to be a transparent, firm, colorless gel, entirely free of microscopic granules such as were found in the ammonia-gelated collagen.[9] Its volume was about 1 ml., of which 98 percent was distilled water. Therefore, to bring it to isotonicity, 1 ml. of double-strength balanced salt solution was added to the tube, which was then stoppered and placed in the roller drum at at 37° C.[10] Within a very few minutes, the free fluid content of the tube was found to be markedly increased, indicating rapid syneresis of the gel. Within 24 hours the gel was flattened and smooth, its rounded edges blending optically with the glass. After removal of the syneresed fluid, medium containing a high concentration (70%) of serum was added and allowed to equilibrate for at least 24 hours with the balanced salt solution in the gel. During equilibration with serum, syneresis slowly continued, and the gel became tougher. After that, it was ready for tissue implantation. The old medium was removed from this tube, fresh medium added, and the tissue fragments were gently placed on the gel surface. After allowing the tube to stand upright for 15 to 60 minutes, it was placed in the roller drum for incubation. Raveling and slippage of the collagen gel sometimes occurred during the cultivation of cells on it, but this was never frequent enough to be a major problem. Although ammonia-gelated collagen and dried collagen films were tested in a few cases, most of this survey was carried out using the dialyzed collagen gel.

Cell Strains Tested and Evaluation of Response on Collagen

Of 29 strains or tissues tested, 24 were of human origin, 2 were rat-fibroblast strains, and 3 were fresh explants from frog tissues. These tissues are classified in table 1. Although a wide variety of tissues were studied, in some cases only a few cultures were available. Because of their dramatic response to collagen, three of these strains were studied more intensively than the others, namely, the altered rat fibroblasts (strain *14p-L*), normal kidney fibroblasts (strain *RoHa*), and the slow-growing carcinoma of the cervix (strain *ElCu*). In all but four cases, growth on collagen was compared to growth of the same tissues simul-

[9] See Addendum.

[10] In a few cases, after placing the disc in a roller tube, it was allowed to dry out completely. Invariably, it dried into a retracted, wrinkled film which proved unsatisfactory for cell-culture work, even when wet again with culture medium. This difficulty was not encountered when gel discs were dried on coverslips for use in drilled slide cultures.

taneously set up on the glass wall of roller tubes, usually in the same tube containing the collagen. In the four exceptions, the controls on glass were set up prior to the experimental tubes. Observations were made over periods ranging from 1 week to 3 months, with particular attention paid to extent of outgrowth and degree of outgrowth retraction. The ability to make successful collagen-to-collagen subcultures was evaluated in 10 of the strains. See table 1 for media used in these experiments.

TABLE 1.—*Cell strains and tissues tested on collagen gels*

Cell strains and tissues	Age and sex of patient	Cultivation prior to collagen testing	Culture medium*
Cell strains previously showing good growth capacity			
Rat fibroblasts (*14p*)	—	17 yrs.	A
Altered† subline of above strain (*14p-L*)	—	—	A
Epidermoid carcinoma of cervix (*HeLa*)	30 yrs., F	4 yrs.	B
Cell strains previously showing moderate growth capacity (human)			
Epidermoid carcinoma of cervix (*ElCu*)	51 yrs., F	3 yrs.	B
Embryonic heart fibroblasts (*MaCr*)	32 mm. embryo	5½ mos.	C
Cell strains previously showing poor growth capacity (human)			
Embryonic skin-muscle fibroblasts (*MaCr*)	"	"	C
Embryonic spinal cord, mixed-cell types (*MaCr*)	"	"	B
Embryonic iris epithelium (*MaCr*)	"	"	B
Embryonic iris fibroblasts (*MaCr*)	"	"	B
Nasal mucosa fibroblasts (*DaPo*)	14 yrs., F	5 mos.	D
Cartilage fibroblasts (*JeRe-N*)	41 yrs., M	3¾ yrs.	B
Kidney fibroblasts (*RoHa*)	2½ yrs., M	9 mos.	A
Kidney fibroblasts (*JoBe*)	10 yrs., F	1 yr.	C
Kidney fibroblasts (*CaRh-N*)	71 yrs., F	11 mos.	B
Kidney fibroblasts and epithelium (*JaJo-N*)	59 yrs., M	5 wks.	B
Kidney papilloma epithelium, benign (*MaLo*)	77 yrs., F	7 mos.	B
Fresh explants of human tissue			
Intraepithelial cervical carcinoma (*MaTho*). Fibroblasts	64 yrs., F	None	A, B, E
Adenocarcinoma of kidney (*LeGl-T*). Fibroblasts	51 yrs., M	2 days	B
Normal tissue from same kidney (*LeGl-N*). Epithelium	"	"	B
Hypernephroma of kidney (*LeHu-T*). Fibroblasts	33 yrs., M	1 mo.	F
Normal tissue from same kidney (*LeHu-N*). Fibroblasts	"	"	B
Metastasis to liver from hypernephroma (*JoCh*). Fibroblasts, macrophages, tumor cells (?)	64 yrs., M	1 day	B
Wilms's tumor of kidney (*RbHn-T*). Fibroblasts	12 yrs., M	"	B
Normal tissue from same kidney (*RbHn-N*). Epithelium	"	"	B
Wilms's tumor of kidney (*JuBr-T*). Fibroblasts	2 yrs., F	9 days	B, G
Normal tissue from same kidney (*JuBr-N*). Fibroblasts and epithelium	"	"	B, G

See footnotes at end of table.

TABLE 1.—*Cell strains and tissues tested on collagen gels*—Continued

Cell strains and tissues	Age and sex of patient	Cultivation prior to collagen testing	Culture medium*
Fresh explants of frog tissue			
Skin. Epithelial growth on glass only....	—	None	H
Kidney. Epithelial outgrowth.....	—	None	H
Urinary bladder. Epithelial outgrowth on collagen only.....	—	None	H

*Explanation of medium symbols:
A=40% BSS (Gey), 10% beef embryo extract, 50% human cord serum.
B=50% BSS, 50% human cord serum.
C=40% BSS, 60% human cord serum.
D=50% Parker's Medium 858 (courtesy of Connaught Laboratories, Toronto, Canada) (*45*), 50% human cord serum.
E=40% Parker's Medium 199 (courtesy of Microbiological Associates, Inc., Bethesda, Md.) (*46*), 10% bee embryo extract, 50% human cord serum.
F=50% Eagle's Basal Medium (courtesy of Microbiological Associates, Inc., Bethesda, Md.) (*47*), 50% human cord serum.
G=Same as F, using 80% and 20%, respectively.
H=50% Amphibian Ringer's (*48*), 10% Medium 199 (courtesy of Microbiological Associates, Inc., Bethesda, Md.), 40% human cord serum.

†Alteration took place in the laboratory of Dr. Raymond C. Parker (Dr. Parker, personal communication). See also Anat. Rec. 124: 18, 1956.

Results

All tissues except frog skin showed slight to greatly improved growth on collagen. The predominant feature of this improvement was promotion of outgrowth without the retractions so commonly seen on glass. The more marked the retraction on glass, the more noticeable the improvement on collagen. In many cases, the elimination of retraction was accompanied by a much more extensive outgrowth. In one culture, for example, four 1 mm. kidney fragments covered the entire 1½-inch gel disc with epithelium in 3 weeks, whereas in the control culture the central fragments became detached from the glass and the outgrowths retracted, breaking up into single cells and cell clumps. Cells on collagen had a tendency to elongate and migrate along lines of stress in the gel. The most successful collagen-to-collagen subcultures were obtained with strains which could grow vigorously on glass despite retractions. Those tested were the altered rat fibroblasts (*14p-L*), human embryonic-heart fibroblasts (*MaCr*), and cervical carcinoma (*HeLa*). There was every indication that these three strains could be carried indefinitely through successive subculturing on collagen. There is evidence accumulating that the slow-growing cervical-carcinoma strain (*ElCu*) can also be subcultured indefinitely on collagen, the best results being obtained when the tissue is scraped free of the old collagen before transfer.[11] In the following strains, cells migrated but failed to proliferate extensively in collagen subcultures: human embryonic-iris epithelium and fibroblasts (*MaCr*), cervical carcinoma (*MaTho*), human cartilage (*JeRe-N*), kidney epithelium

[11] See Addendum.

(*RbHn-N* and *LeGl-N*), Wilms's tumor fibroblasts (*RbHn-T*), kidney fibroblasts (*LeHu-N*), and hypernephroma fibroblasts (*LeHu-T*).

None of the strains tested caused any visible lysis of the collagen substrate, in this respect placing collagen at an advantage over the traditional plasma clot. With the exception of the *14p-L* and *HeLa* strains, both of which often grow on glass without retraction [*e.g.*, (*49*)] the strains selected for study on collagen presented a growth problem in that they consistently showed moderate to severe retraction on glass. Details of the response on collagen can best be shown by illustrated examples.

Figure 2 compares the gross appearance of two living roller-tube cultures of strain *14p-L* rat fibroblasts 5 days after transfer. The colonies in the top tube are growing on a dialyzed collagen gel, the edge of which is faintly visible, whereas in the bottom tube they are growing on the bare glass wall. On collagen, the colonies have continuous, spreading outgrowths, whereas on glass one can easily see retractions with resultant islands of clumped material, which prove to be cell clumps under the microscope. The missing colony is visible at the lower border of the tube, where it slipped a few hours after explantation. Such slippage of explanted fragments is far less frequent on collagen than on glass. In this strain of cells, *p*H studies of the medium and microscopic examination indicate that colony size is increased on collagen because of greater spread rather than the presence of more cells.

Figures 5*a* and 5*b*, showing 6-day-old *14p-L* colonies on collagen and on glass, respectively, illustrate this effect quite well. On collagen, the outgrowth is well spread out, while on glass it is growing as a retracted mass of densely packed cells. This particular cell strain is vigorous enough to grow despite severe retractions on glass, whereas in other cell types, such as human kidney epithelium and strain *ElCu* cervical carcinoma, such retractions lead to a complete cessation of growth.

Figures 3*a* and 3*b* again give evidence of the response to collagen of *14p-L* rat-fibroblast colonies in a roller tube prepared with a thin coat of ammonia-gelated collagen. The tube was wet with acetic acid-collagen solution and then inverted on a cotton plug moistened with ammonia. In a few minutes, the wet surface jelled. The tissue fragments were placed in the tube after the gel coat had been washed and equilibrated with medium. Like the dialyzed gel, outgrowth on the coated-tube surface is continuous and well spread out, whereas on the bare glass wall, retraction and fragmentation are severe, with individual cells as well as masses of cells undergoing retraction. Coating tubes with collagen solution, as just described, is not recommended as a general practice, because the coat often tears and ravels.[12]

Figures 4*a* and 4*b* show portions of the same culture at higher magnification. Cell-surface pseudopodia in figure 4*b* stand out prominently due to off-axis illumination, in this case accidentally resulting from refraction of light by the central fragment.

As described above, collagen gel prevents fragmentation and collapse of outgrowth in strain *14p-L* rat fibroblasts. Other related casualties com-

[12] See Addendum.

monly occurring on glass are also prevented by collagen, and will be described in connection with figures 6, 7, and 8. Holes and breaks often appear in previously intact outgrowths of many different cell types. As described so aptly by Hitchcock (*50*) for frog epithelium, "The holes enlarge rapidly as outgrowth continues, and soon the explant and outgrowth are connected only by narrow strands which become pulled out into extremely attenuate filaments that finally rupture. The outgrowth now appears like a halo about the explant." Illustrative examples of this type of effect are found in figures 6*b*, 15*b*, and 19*b*.

In figure 6*b*, outgrowth and central fragment are separating in a *14p-L* rat-fibroblast colony when grown for 10 days in Parker's synthetic mixture No. 858 (*45*) in the absence of serum. In the same medium, the experimental colony on collagen (fig. 6*a*) shows no sign of a break.

Cell strains vary widely in their behavior on glass, depending on the ability of their outgrowths to remain on the glass without retracting and on their growth capacity. Some strains, such as the fibroblastic strain from human embryonic spinal cord (*MaCr*) have a low growth capacity, yet their outgrowths adhere well to the glass. This results in tiny stationary colonies which show very little retraction. At the other extreme are strains such as the *14p-L* strain of rat fibroblasts which have a high growth capacity, yet whose outgrowths stick very poorly to the glass. This results in a thick retracted colony which grows despite the retraction. Eventually, chunks of tissue break off, and become washed to other parts of the roller tube where they grow into new colonies. Many strains, such as the *CaRh* strain of human fibroblasts, have a low growth capacity and almost no ability to grow out on the glass. In these cases, the early slight outgrowth retracts as a layer against the central fragment, after which the mass remains quiescent, while scattered cells wander aimlessly about the tube, singly or in small, rounded clumps, and finally die.

An intermediate type of behavior is found in strains such as the 1½-year-old *MaCr* strain of human embryonic-heart fibroblasts (fig. 7*b*), with moderate growth capacity and moderate ability of its outgrowth to maintain itself on glass without retraction. The central fragment retracts while proliferation continues in the outgrowth left on the glass, which gradually fills in the holes and breaks, and spreads slowly outward. There is such a delicate balance between the tendency to proliferate and to retract that this is a difficult strain to grow on glass. However, when grown on collagen (fig. 7*a*), retraction is eliminated and growth is profuse, the central fragment becoming dispersed into the outgrowth. Photographs 7*a* and 7*b* were taken 4 days after transfer. These heart fibroblasts grow unusually well on collagen gel, suggesting a physiologic growth mechanism.

Retraction on glass may start as a localized raveling at the border of an epithelial sheet. This is evident in a photograph of an 8-day-old culture of normal human kidney (fig. 8*b*), strain *RbHn-N*. The border cells are rounded up and crowded together, whereas on collagen (fig. 8*a*), the delicate outgrowth is entirely free of retraction.

Figures 9*a* and 9*b* illustrate a significant accidental finding in a week-old culture on collagen of normal human kidney epithelium (*LeGl-N*) and an 8-day culture on collagen of a fibroblastic strain from a cervical carcinoma (*MaTho*), respectively. In each case, a floating tissue fragment became secondarily attached to the edge of the collagen gel. The photographs show these adherent fragments with a profuse outpouring of cells on the collagen and almost none on the glass.

Figure 10 illustrates the behavior of tissue fragments from a year-old strain of human kidney fibroblasts (*RoHa*) when grown for 6 days on the edge of a collagen gel prepared by exposure to ammonia vapor. A fibroblastic outgrowth spreads down over the collagen layer, whereas almost no growth is seen on the glass. Such a culture showed a repeated pattern of outgrowth, collapse, and regrowth on the glass portion of the tube. The fine background granularity in the collagen layer is typical of ammonia-gelated collagen.[13]

In figure 11 (*see also* figs. 16*a*, 16*b*, and 17) there is fibroblastic outgrowth on collagen, with failure to grow on glass, from a hypernephroma explant (*JoCh*) cultured for 2 weeks on the edge of a dialyzed gel. In contrast to fibroblasts, it will be noted that macrophages migrate profusely over the glass.

Another example, figure 12, shows a month-old epithelial outgrowth on collagen from a fragment of normal human kidney (*LeGl*), grown previously on glass for 6 months. The unbroken mosaic membrane has grown out from the central fragment (lower-left corner), and has reached the edge of the collagen gel, with retracting clumps of cells on the glass beyond.

Similarly, figure 13 shows an 11-day culture of cervical-carcinoma cells (*HeLa*) which have grown as a solid sheet on collagen (left), reaching the border and spilling over onto the glass. This cell strain is capable of growing profusely on glass, even though at times the outgrowth is broken up into isolated cells and small groups as shown here.

Figures 14 and 15 illustrate the dramatic response of a 3-year-old slow-growing strain of cervical carcinoma (*ElCu*) when cultivated on collagen gel. After 19 days' growth on glass (fig. 14*b*), the control colony has retracted and broken up into a few small islands of cells, whereas on collagen (fig. 14*a*) the tissue has grown as a spreading epithelial sheet without retraction. Three weeks later, the collagen gel with its epithelial covering was removed to a petri dish, cut into small fragments, and retransferred to collagen and to glass. The results are shown in figures 15*a* and 15*b*, 7 days following this second transfer. On collagen (fig. 15*a*) the outgrowth again takes the form of an unbroken epithelial sheet, whereas on glass (fig. 15*b*) expanding holes will soon reduce the outgrowth to islands as in figure 14*b*, with cessation of growth.

Figures 16*a* and 16*b* show different colonies from the same hypernephroma culture described previously (fig. 11), but at a higher magnification—one colony on collagen with fibroblastic outgrowth (fig. 16*a*) and the other on a bare glass portion of the same tube (fig. 16*b*)—showing

[13] See Addendum.

only macrophages. The central fragments are partially visible at the lower-left corners of the photographs. In addition to fibroblasts and macrophages, some colonies on collagen had masses of huge, moderately vacuolated cells (fig. 17), which never showed mitoses. It was felt that these cells were the true hypernephroma tumor cells. The largest in the photograph measures 0.3 mm. in length.

A 2-day-old culture of a Wilms's tumor fragment (*JuBr-T*) on collagen gel (fig. 18*a*) shows profuse fibroblastic outgrowth, in contrast to the loose retracted growth on bare glass in the same tube (fig. 18*b*), the central fragments again pictured in the lower-left corners.

Fixing these or other strains of cells on collagen gel with methyl alcohol and staining with Giemsa gives excellent results, as seen in figures 19*a* and 19*b*. These show 4-day-old Wilms's tumor fibroblast colonies (*JuBr-T*) on collagen gel and glass, respectively. The cells are violet with blue nuclei, whereas the collagen gel remains transparent, with only a faint pink hue. Hematoxylin was found unsatisfactory as a stain for cells on collagen gel.

Discussion

The purpose in presenting the foregoing results has been to show that rat collagen, reconstituted as a clear gel, promotes a cellular outgrowth usually free from retraction. Certain advantages of dialyzed collagen gel over plasma clot [used without heated serum or inhibitor (*12–14*)] should be noted. Firstly, the collagen gel is not lysed by the cultured cells of this series. Secondly, in contrast to plasma clot, cells remain on the gel surface, thus maintaining intimate contact between cells and fluid medium. One disadvantage of the collagen gel is the rather cumbersome technique required for its preparation, which can perhaps be simplified.[14] Some observations suggest that certain cell strains may grow more profusely on collagen than in clot, namely, a slow-growing strain of normal human cervix (*ViSo*, not included in table 1) and 2 strains of cervical carcinoma (*MaTho* and *ElCu*). In a strain of normal human cartilage (*JeRe*) there was some indication that growth was better in plasma clot than on collagen. Other than these, too few observations have been made to evaluate collagen in terms of plasma clot.

The nature of the response to collagen has not been determined, although a mechanical mode of action is suggested. For example, when the kidney epithelium pictured in figure 12 was subjected to digestion by 0.5 percent trypsin for 40 minutes, the only cells which rounded up and loosened were those located on the bare glass. On collagen, the membrane of cells remained intact, indicating a strong adherence of cells to the underlying gel. Perhaps the cells become firmly rooted by means of pseudopodia. Electron-micrographic studies confirm this. Some findings suggest that collagen gel also has chemical and nutritional effects on cells. This would not be surprising, since the gel used in this study was a crude preparation which may contain other substances besides collagen. For example, toxic effects were occasionally seen, such as transient increases

[14] See Addendum.

in cell granularity,[15] an initial growth inhibition of human embryonic-heart fibroblasts (fig. 7) soon followed by exceptionally profuse growth for that strain, a transient initial inhibition of *14p-L* rat fibroblasts when grown on collagen in synthetic medium 858 without serum (fig. 6*a*), and a failure of frog (*R. pipiens*) skin epithelium to grow on rat collagen despite growth on glass. Some or all of these occasional toxic effects might have resulted from incomplete equilibration of collagen with culture medium prior to explantation. In contrast to possible toxic effects, a few observations suggest that the collagen gel may be of nutritional benefit to cells, either by direct stimulation or by neutralization of a toxic component in serum. For example, in a culture of human embryonic-heart fibroblasts on collagen, cells on the glass portion grew much more rapidly than in cultures not containing collagen.[16] When the collagen was removed, growth continued rapidly for a short while and then slowed down. This reminds one of the effect of adding bits of cartilage to tissue cultures, prolonging the life of chick fibroblasts in synthetic medium (*45*), or improving morphogenesis of embryonic tissues (*51*). In the first instance, growth stimulation was probably due to the glucuronic acid present in the cartilage (*45*). In figure 8*b*, vacuolated cells are present on the retracting border of kidney epithelium on glass but absent along the intact border on collagen (fig. 8*a*). This happens so regularly when kidney epithelium is grown on collagen as to suggest a protective effect. Other observations relative to these findings come from studies on various cell strains in which similar lessening of retraction and degeneration has been obtained by lowering the serum concentration, *e.g.*, figure 4 in the publication by Glinos and Gey (*52*), and the observation by Carrel and Ebeling that increased serum concentrations shortened the life span of fibroblasts (*53*). Thus, we see that the beneficial effect of collagen gel on cell growth may be due to a combination of factors.

Actually, little is known about the relationships between collagen and cells. Both collagen, as reviewed by S. Fitton Jackson (*54*), and mucopolysaccharides (*55,56*) are produced by cells *in vitro* and may form a layer over which cells can migrate, similar to the "ground mat" described by Weiss (*7*). Colonies may become embedded in a mucoid matrix of these substances. Other strains such as the *MaCr* strain of human embryonic-heart fibroblasts, exude a thin transparent layer on the glass wall of the culture vessel over which the cells grow and become attached. That the matrix secreted by cells enhances their growth is an attractive theory. Perhaps outgrowth retractions in a given cell strain occur when insufficient matrix is produced, with marked improvement when the deficiency is corrected by growing the strain on a collagen gel. *In vivo* evidence that such a growth mechanism occurs in nature comes from studies of Friedenwald *et al.* (*57*) on the healing of corneal wounds and from the observations of Lash (*58*) on the increased rate of epithelial migration over denuded basement lamellae during the closure of salamander wounds.

[15] Not to be confused with increased deposits of granular debris on the sticky collagen surface
[16] See Addendum.

The resemblance of collagen gel to the natural matrix produced by cells makes it an ideal surface on which to study the physiologic functioning of cells. When softer gels are developed, perhaps by ammonia-gelation techniques, penetrating cells will grow in a 3-dimensional environment which can be experimentally modified to resemble closely many natural and pathologic conditions.

Summary

1) A technique has been described for reconstituting rat-tail collagen as an amorphous transparent gel, slices of which can be placed in roller tubes to serve as a substratum for growing cells in tissue culture.

2) When compared to the bare glass wall of roller tubes, collagen gel proved superior for the growth of 29 cell strains and fresh-tissue explants (human, rat, frog). On collagen gel, colony outgrowth was frequently more extensive, and almost always occurred without the retractions commonly seen on glass.

3) There was no evidence that collagen gel could be lysed by any of the cells or tissues tested, even after prolonged contact.

4) Possible mechanisms of the improved cell response on collagen gel are discussed, as well as its potential value in cell research.

Addendum

Since this publication went to press, we made the encouraging finding that a crystal-clear collagen gel can be produced *in situ* within roller tubes as a slant or as a coating if evaporation or exposure to ammonia vapor is carried out on a partially dialyzed acetic acid-collagen solution. The prior dialysis against distilled water is interrupted as soon as the viscosity of the collagen solution begins to show a marked increase, which usually occurs after 1 to 3 days. This thickened solution can be stored in the deep freeze, but centrifugation may then be necessary to remove tiny bubbles which form during thawing. To prepare a roller tube, 1 or 2 ml. of the partially dialyzed collagen solution are placed into it. For rapid gelation, the tube is plugged with ammonia-moistened cotton and laid on its side. In a few minutes, the solution gels, but remains perfectly clear. If, instead, the horizontal tube is rotated about its long axis, the ammonia vapor gelates the solution as a transparent coating. The only drawback to these ammonia gels is the necessity of washing them free of ammonia (using 0.002% phenol red as indicator in the distilled water) before equilibrating with double balanced salt solution, as described in the paper. If, instead of exposing to ammonia vapor, the roller tube is simply plugged loosely and laid on its side for 2 or 3 days at room temperature, the partially dialyzed collagen solution will gel spontaneously. Although not yet tried, one would expect a gel coat to be formed if the tube were rotated during this period. If evaporation is carried to completion, the gel dries down to an invisible film. These new simplified techniques show considerable promise for use in tissue culture.

In addition to these new findings, initial studies indicate that transfer

of cells cultured on collagen can easily be made after dissolving the collagen gel with 1 or 2 mg. percent collagenase (courtesy of Dr. John D. MacLennan, College of Physicians and Surgeons, Columbia University, New York).

Regarding the possible nutritive role of these collagen preparations in tissue cultures, Dr. H. Naim Kent in this laboratory finds that a slice of dialyzed collagen gel (without cells), when incubated at 37° C. for 3 days with balanced salt solution, releases trace amounts of amino acids into the salt solution.

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PLATES

379436—56——9

PLATE 122

FIGURE 1.—Dried transparent collagen membrane. × 1

FIGURE 2.—Five-day-old living roller-tube cultures of strain *14p-L* rat fibroblasts on dialyzed collagen gel (*above*) and on glass (*below*). × 6

This is a sheet of reconstituted amorphous collagen obtained from 80cc of a 1:1,400 acetic acid extract of rat tail tendons. The extract was poured into a petri dish, covered with a sheet of cellophane, and dialyzed for 10 days against distilled water. The resultant clear gel was dried for 4 days on cellophane stretched across a metal hoop. Then, under water, the collagen membrane was loosened from the cellophane, stretched across another metal hoop, and re-dried in the open air. Wrinkling resulted from loss of tension due to the membrane tearing, and pulling loose from the hoop during drying.

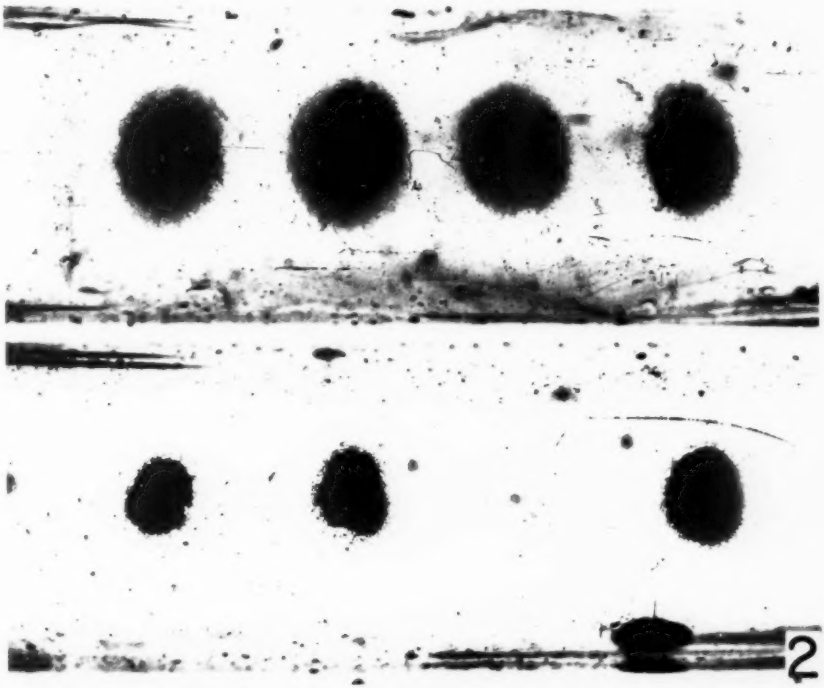

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PLATE 123

FIGURE 3.—*a*) Portion of a living *14p-L* rat-fibroblast colony after 13 days' growth in a roller tube coated with ammonia-gelated collagen. × 39

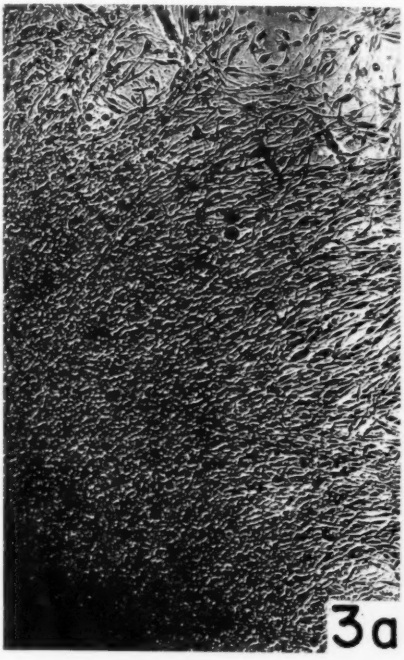
b) Control colony on glass. × 39

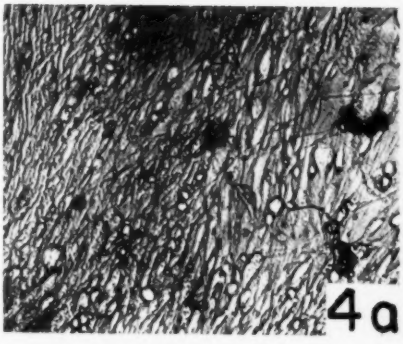
FIGURE 4.—*a*) A higher magnification of colony in figure 3*a*. × 104

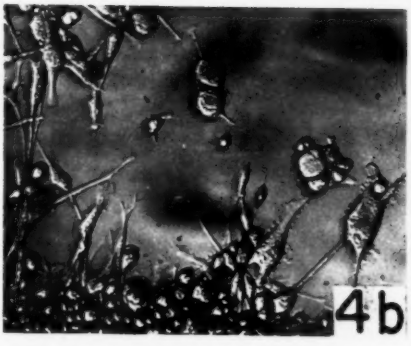
b) A higher magnification of colony in figure 3*b*. × 104

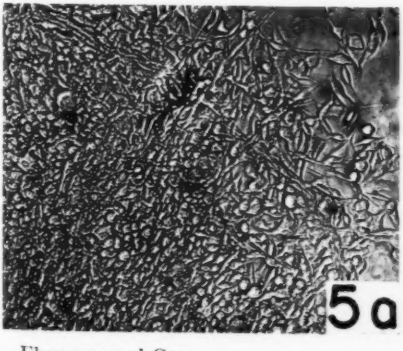
FIGURE 5.—*a*) Portion of a living *14p-L* rat-fibroblast colony after 6 days of growth on a dialyzed collagen gel. × 86

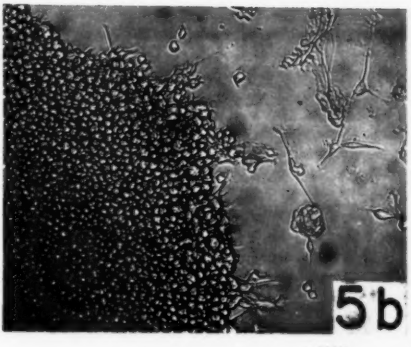
b) Control colony on glass. × 86











Ehrmann and Gey

379436—56——11

PLATE 124

FIGURE 6.—*a*) Portion of a living *14p-L* rat-fibroblast colony in Parker's Medium 858 after 10 days of growth on a dialyzed collagen gel. × 40

b) Control colony on glass. × 40

FIGURE 7.—*a*) Portion of a living human embryonic-heart-fibroblast colony (*MaCr*) after 4 days of growth on a dialyzed collagen gel. × 39

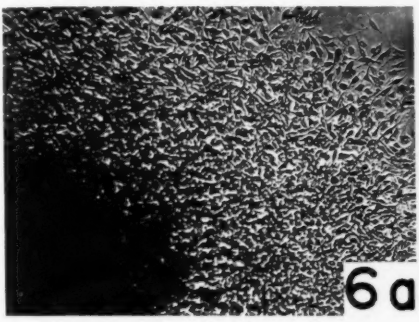
b) Control colony on glass. × 39

FIGURE 8.—*a*) Edge of outgrowth from a fragment of human kidney (*RbHn-N*), 7 days after transfer to a dialyzed collagen gel. × 163

b) Edge of outgrowth from control fragment on glass, 8 days after transfer. × 163

FIGURE 9.—*a*) Growth of cells only on the collagen and not on the adjacent glass from a fragment of human kidney (*LeGl*) adherent to the edge of a dialyzed collagen gel, 7 days following transfer. × 58

b) Similar selective growth only on collagen from a fragment of human cervical carcinoma (*MaThe*), with no growth on the adjacent glass, 8 days following transfer. × 34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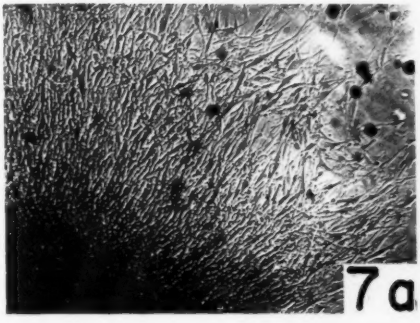
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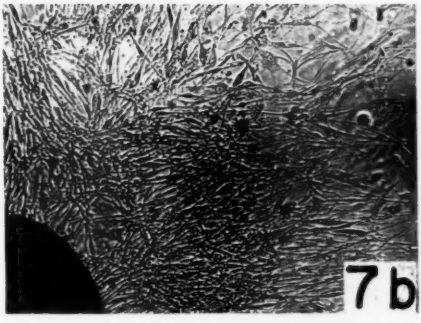
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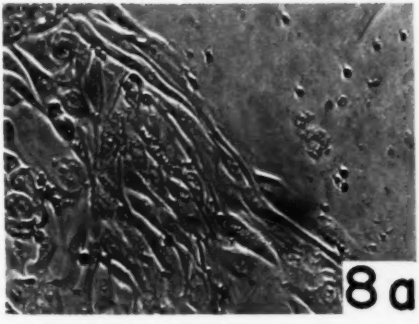
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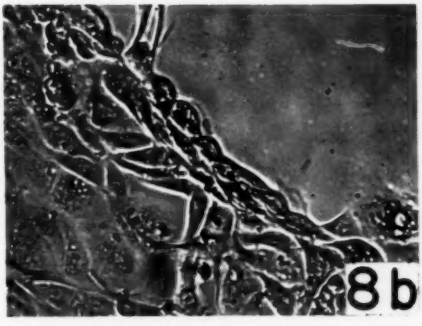
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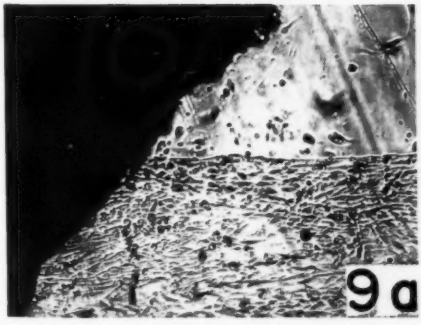
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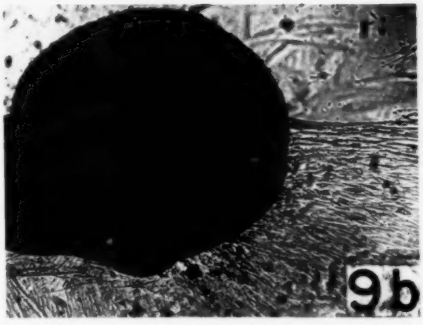
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PLATE 125

FIGURE 10.—Living colony of human kidney fibroblasts (*RoHa*) after 6 days of growth on the edge of a collagen gel prepared with ammonia vapor, with growth only on collagen and not on glass. × 50

FIGURE 11.—Outgrowth of fibroblasts on collagen and macrophages on glass from a hypernephroma liver-metastasis fragment (*JoCh*) 14 days after transfer to the edge of a dialyzed collagen gel. × 40 (*See also* figs. 16*a* and 16*b*.)

FIGURE 12.—Human kidney epithelium (*LeGl-N*) on edge of dialyzed collagen gel with slight spread to glass beyond, 1 month after transfer. Central fragment is visible at *lower-left corner*. × 103

FIGURE 13.—Cells from strain of human cervical carcinoma (*HeLa*) growing on edge of dialyzed collagen gel (*left*) with spread to glass (*right*) 11 days following transfer. × 92

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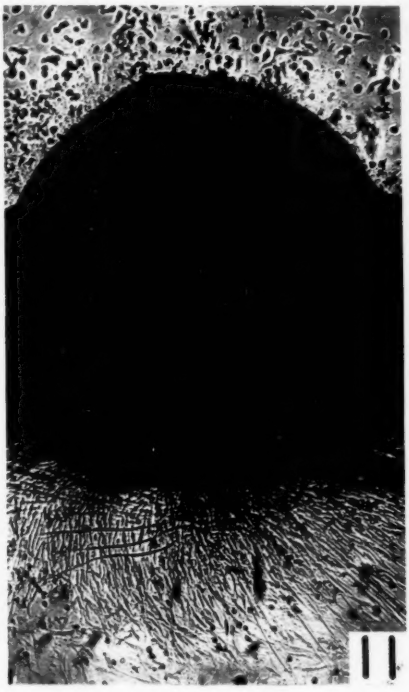
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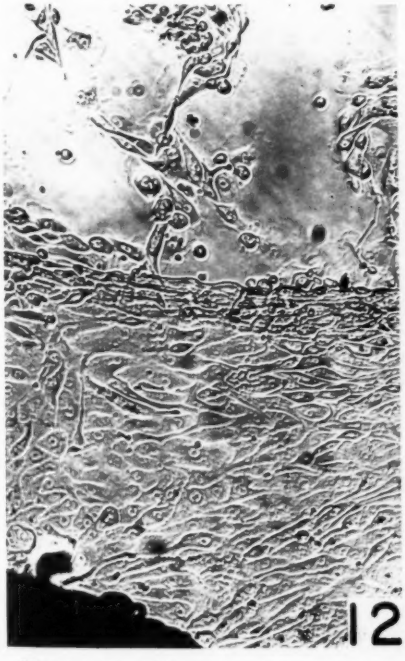
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PLATE 126

FIGURE 14.—*a*) Living membrane of human cervical-carcinoma cells (strain *ElCa*) growing on a dialyzed collagen gel 19 days after transfer. × 104

b) Control culture on glass showing severe retraction of membrane. × 104

FIGURE 15.—*a*) Subculture from *ElCa* culture shown in figure 14*a* to another tube containing a dialyzed collagen gel, 7 days after transfer to this second piece of collagen. × 104

b) Control subculture from *ElCa* culture shown in figure 14*a* to the glass wall of another tube. × 104

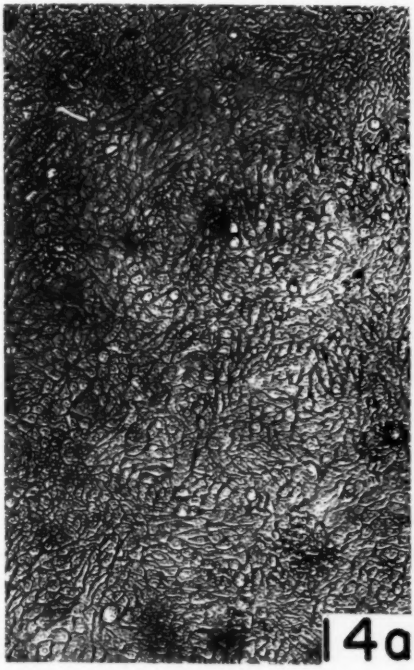
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PLATE 127

FIGURE 16.—*a*) Portion of a living colony from a human hypernephroma liver metastasis (*JoCh*) on a dialyzed collagen gel 5 days after transfer, showing fibroblastic outgrowth and an isolated vacuolated tumor (?) cell. × 90

b) Control colony on glass portion of same tube, showing only macrophages. × 90 (*See also* fig. 11.)

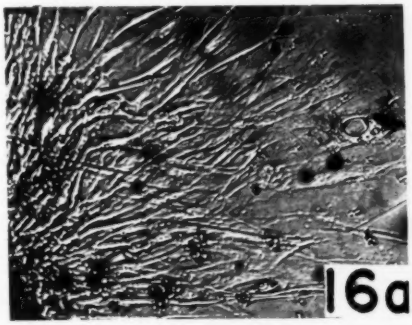
FIGURE 17.—Same culture 14 days after transfer, showing an area of tumor (?) cells on collagen. × 107

FIGURE 18.—*a*) Portion of a living colony from a Wilms's tumor of the kidney (*JuBr-T*) 2 days after transfer to a dialyzed collagen gel, showing fibroblastic outgrowth. × 104

b) Control colony on glass portion of same tube. × 104

FIGURE 19.—*a*) Portion of colony from same Wilms's tumor (*JuBr-T*) grown on a dialyzed collagen gel for 4 days, then fixed with methyl alcohol and stained with Giemsa. × 69

b) Control colony on glass portion of same tube, fixed with methyl alcohol and stained with Giemsa. × 69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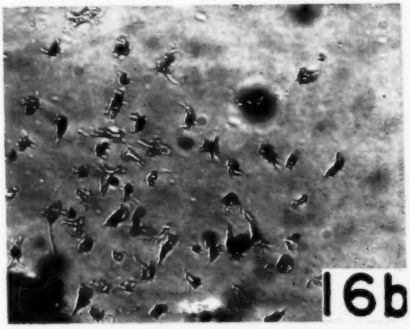
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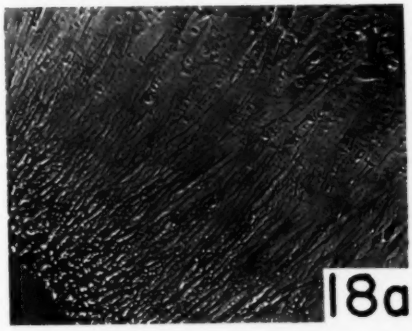
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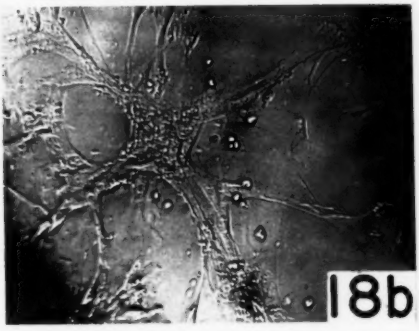
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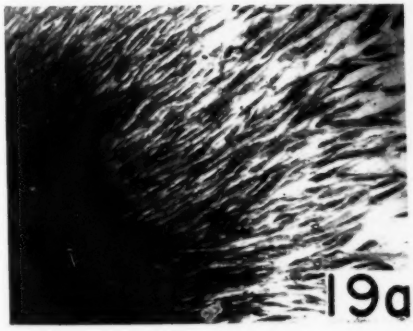
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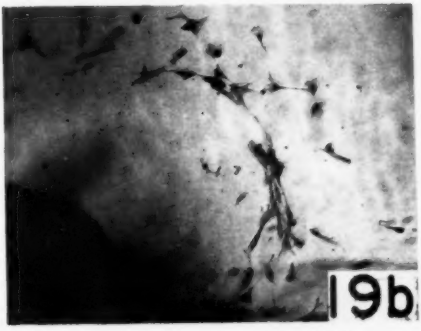
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The Nutrition of Animal Tissues Cultivated *in Vitro*. II. A Comparison of Various Synthetic Media[1]

JOSEPH F. MORGAN, HELEN J. MORTON, M. ELIZABETH CAMPBELL, *and* LAURENCE F. GUERIN,[2] *Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada*

Current interest in cell nutrition and physiology has stimulated intensive efforts to devise media of chemically known composition that will support the survival and propagation of animal tissues cultivated *in vitro*. As a result of these efforts, a number of media have been proposed for supplementation of dialyzed natural media (*1,2*), for prolonged maintenance of cell survival (*3,4*), or for rapid, short-term cell multiplication (*5*). In the development of these media, a variety of culture techniques were employed but no comparative evaluation, using one standardized type of tissue culture, has yet been reported. Consequently, it was considered of interest to make such a comparison in the hope that the nutritional adequacy of the various synthetic media could then be related to the presence or absence of specific growth factors. To avoid the possibility of cell adaptation to the different media through repeated subcultivation, fresh tissue explants, rather than established cell strains, were employed in this study, using one standard technique based on the determination of cell survival.

Previous publications from this laboratory have demonstrated the suitability of this culture system for measuring the response curves of certain essential amino acids (*6*) and natural materials (*7*) and have established the statistical significance of small differences in survival times (*6*). The results reported in this communication show the great variation that exists in the nutritional value of the present synthetic media and indicate that this variation can be related to both the culture system employed and the specific composition of the experimental media.

Materials and Methods

All cultures were prepared from the heart muscle of 11-day-old chick embryos. The tissue was chopped to a fine pulp and moistened with a few drops of synthetic medium, and portions were transferred to 18 × 150 mm. pyrex test tubes by a capillary pipette. Cultures prepared in this way contained from 1 to 2 mg. of tissue, wet weight, as determined by fre-

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quent weighings of replicate samples and by total-protein determinations (*8*). After a 25- to 30-minute period, during which the tissue became fixed to the glass without the use of plasma clots, 0.75 ml. of synthetic media were added and the cultures placed in stationary roller drums with the medium covering the tissue. After 24 hours' stationary incubation at 37° C., the drums were started and rotation was maintained at 12 r.p.h. for the duration of the experiment. All cultures were examined microscopically 7 days after preparation, to estimate the extent of proliferation and the general health of the cells. Following this initial appraisal, the cultures were divided into uniform groups of 12 to 30 cultures, the starting medium was removed, and 1.5 ml. of the test media were added. These media were removed and replaced twice a week for the duration of the experiment. Each culture was examined microscopically at frequent intervals until living cells could no longer be seen.

In the presentation of the experimental results, cumulative average survival for the cultures in each test medium has been calculated. Statistical analyses of the data have been made by the alternate *t* test, which is based on unequal variances in the populations being compared. These analyses have established that all differences in survival times discussed in this report are significant at the 1 percent level. The standard error of each mean survival time has also been calculated and embodied in the text.

The basic synthetic medium employed was M 150 (modified 199) prepared as described in a previous communication (*7*). Other synthetic media tested were S-27 of Rosenberg and Kirk (*2*), prepared in Hanks' balanced salt solution (*9*), V-614 of Fischer, Astrup, Ehrensvärd, and Øhlenschläger (*1*), White's medium (*10*) and No. 703 of Healy, Fisher, and Parker (*5*), prepared in Hanks' solution. In addition to these, a new medium was prepared (M 416) which was based on the formula of Healy's 703 but which contained purines and pyrimidines, adenosinetriphosphate (ATP), and adenylic acid. These ingredients were present, at the same concentrations, in the original formula of medium 199 (*4*) but were omitted from the modification which resulted in medium 703 (*5*). Synthetic medium M 150, prepared without sodium bicarbonate and used for moistening the tissue pulp, was designated M 161. The prefix "M" has been used to designate media developed in this laboratory. Since M 1 followed solution 586 in the original series (*4*), M 416 represents the development of more than 1,000 different synthetic media. The composition of the various synthetic media studied has been arranged in order of decreasing complexity and is summarized in table 1.

All chemicals employed were of the highest grade obtainable commercially and were dissolved in ion-exchange water (*11*) to prepare the required stock solutions. All synthetic media were sterilized by filtration through UF fritted-glass filters.

Results

Preparation of uniform cultures.—Earlier studies on the preparation of roller-tube cultures of chick embryonic tissues (*12*) established that such

TABLE 1.—*Composition of the various synthetic media studied*

Medium	Composition
Morgan's M 416.....	Coenzymes; high levels of cysteine, ascorbic acid, and glutathione; purines and pyrimidines, ATP, adenylic acid; 19 amino acids; B vitamins, fat-soluble vitamins; glutamine, acetate.
Morgan's M 150*.....	Low levels of cysteine, ascorbic acid, and glutathione; purines and pyrimidines, ATP, adenylic acid; 19 amino acids; B vitamins, fat-soluble vitamins; glutamine, acetate.
Healy's 703.....	Coenzymes; high levels of cysteine, ascorbic acid, and glutathione; 19 amino acids; B vitamins, fat-soluble vitamins; glutamine, acetate.
White's medium.....	Low levels of cysteine, ascorbic acid, and glutathione; 15 amino acids; B vitamins, B_{12}, fat-soluble vitamins.
Fischer's V-614.....	Ten amino acids; glutathione, glutamine, hexose diphosphate.
Rosenberg's S-27.....	Two amino acids; 1 purine, 1 pyrimidine; hexose diphosphate, B_{12}, folic acid.

*Medium M 150, prepared in Hanks' salt solution without sodium bicarbonate and used to moisten the tissue pulp, is designated M 161.

cultures could be started directly in the synthetic medium, provided the volume of medium was reduced to half the normal quantity and was left without change for 5 to 7 days. Even under these conditions, many fragments failed to adhere to the glass and the cultures were frequently too irregular for use. For this reason, a detailed study was made of the factors influencing the adhesion of the tissue fragments to the glass surface.

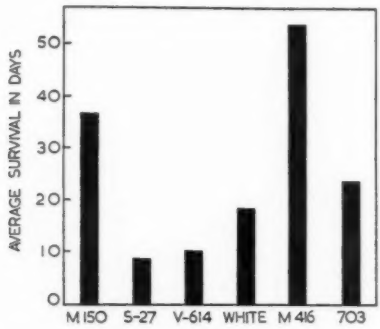
Prolonging the period of contact of the tissues with the glass before adding the synthetic medium did not increase adhesion but rather caused excessive drying and death of the fragments. Attempts were therefore made to modify the synthetic medium used to moisten the tissue pulp before the preparation of cultures. Medium M 150 was prepared and to it were added graded concentrations of Tween 80, methocel (4,000 centipoise), glycerol, or glucose. Medium M 150 was also prepared with bicarbonate omitted (M 161).

It was found that incorporation of 0.25 percent Tween 80, 1.0 percent methocel, or 20 percent glycerol markedly increased the adhesion of the tissue fragments, yielded more uniform cultures, and prolonged the survival of the cultures to a moderate degree. M 161 was equally satisfactory. Lower concentrations of Tween, methocel, glucose, or glycerol were less effective, and high concentrations of glucose were toxic. Since the omission of bicarbonate was a simple and effective procedure, M 161 was used as the "wetting medium" in all further experiments.

It should be emphasized that these studies were concerned only with the medium used to moisten the tissue pulp. Once the fragments became fixed to the glass (25 to 30 minutes), the tissue was bathed in medium M 150, and considerable dilution of the wetting medium occurred. This dilution was greatly increased during the subsequent twice weekly changes of

M 150. The magnitude of this dilution made it possible to employ a concentration of Tween 80 (0.25%) in the concentration range (0.5 to 0.05%) which had been shown to be toxic during prolonged cultivation (*13*).

Relative survival of chick embryonic-heart fragments cultivated in representative synthetic media.—Groups of twelve to fifteen 11-day-old chick-embryo hearts were pooled, chopped to a fine pulp, and moistened with 0.1 ml. of medium M 161. From these tissue pools, lots of 80 relatively uniform cultures were prepared and incubated for 7 days in medium M 150. After microscopic evaluation, these cultures were subdivided into similar groups of 15 to 20 cultures, the M 150 was removed, and the various synthetic media were added. These experimental media were removed and replaced twice a week until death of the cultures. The relative survival times of these cultures in various synthetic media, summarized from a large number of experiments, are portrayed in text-figure 1.

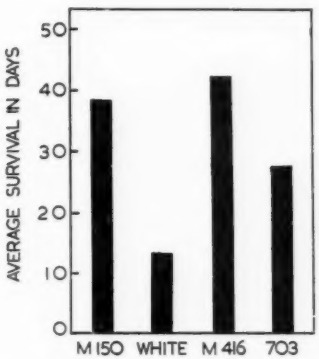


TEXT-FIGURE 1.—Relative survival time of freshly explanted chick embryonic-heart tissues cultivated in various synthetic media after initial 7 days' cultivation in synthetic medium M 150.

The varying ability of these representative media to support the survival of freshly explanted chick embryonic-heart tissues is clearly shown. The poorest survivals (9.0 ± 0.4 and 9.8 ± 4.2) were obtained with Rosenberg's S-27 and Fischer's V-614, respectively. White's medium (18.0 ± 7.6) and Healy's 703 (26.0 ± 18.9) supported an intermediate length of survival. The most suitable media were found to be M 150 (35.8 ± 12.8) and M 416 (53.6 ± 22.4).

Experiments were carried out to determine whether the initial 7 days' cultivation in medium M 150 had altered the response of the tissues during their subsequent cultivation in the different media. Large sets of cultures were prepared from chick embryonic-heart pulp moistened with M 161, divided into groups at random, supplied immediately with the test media, and maintained in these media until death. The survival times obtained in these experiments were in close agreement with those recorded previously (text-fig. 1). It would appear, then, that the relative suitability of the various media tested was not influenced appreciably by the preliminary cultivation in medium M 150.

Further experiments were carried out to determine whether moistening the tissue pulp with medium M 161 influenced the final survival time of the cultures in the various test media. Large pools of embryonic-heart-tissue pulp were prepared in the usual way and divided into four portions; then individual portions were moistened with either M 150, M 416, Healy's 703, or White's medium. Cultures were then prepared from these four tissue samples and maintained in the same four media until death. The results of these experiments are shown in text-figure 2. It is apparent that starting the cultures in the individual media and maintaining them in the same media has not appreciably altered the length of the culture survival. The two best media were still found to be M 416 (43.6 $\pm$ 8.9) and M 150 (38.0 $\pm$ 9.9). The period of survival in 703 (28.5 $\pm$ 18.1) was essentially unaltered from the previous value, but White's medium (12.1 $\pm$ 2.1) showed a marked decrease in survival time.



TEXT-FIGURE 2.—Relative survival time of freshly explanted chick embryonic-heart tissues moistened with various synthetic media and then cultivated in these same media.

Comparative appearance of chick embryonic-heart cultures in various synthetic media after initial 7-day cultivation period.—Cultures prepared from tissue samples moistened with the various test media and then cultivated directly in these same media showed striking differences in extent of migration and health of cells when examined microscopically at the end of the initial 7 days' cultivation. Photomicrographs of a typical culture in each test medium are shown in figures 1 to 6. The magnification in these photomicrographs ($\times$42) has been selected to portray the cultures as a whole, rather than the fine detail of individual areas.

Cultures in the control medium, M 150 (fig. 1), show a typical dense sheet of healthy spindle cells migrating a considerable distance from the central fragment. The edge of this central fragment exhibits a gradation in density associated with active cell migration and the presence of several cell layers. Cultures in Rosenberg and Kirk's S-27 (fig. 2) were dying at the end of 7 days, and only a few tiny rounded cells can be seen at the edge of the central fragment. The appearance of cultures in Fischer's V-614

(fig. 3) is characterized by scattered migration of small, narrow, retracted, starved cells with no confluent sheets. Cultures in White's medium (fig. 4) show a moderate degree of cell migration. Although the extent of migration is not great, the individual cells showed the typical spindle shape of healthy fibroblasts. These sheets of healthy cells, however, did not persist but degenerated and died quite quickly. Cultures in medium M 416 (fig. 5) are characterized by extensive migration of confluent sheets of spindle-shaped cells, associated with marked gradation in density of the central fragment. The epithelium-like appearance of one area is due to the presence of more than one cell layer and subsequent loss of cell detail in reproduction. The appearance of these cultures is essentially similar to that of cultures in M 150. Cultures in Healy's 703 (fig. 6) show scattered cells and irregular sheets. The cells are spindle-shaped but rather small.

There is good agreement between the appearance of the cultures after 7 days' cultivation in various media, as shown in plate 128, and the relative survival time of cultures in the same media (text-figs. 1 and 2). Over a 2-year period, more than 90 percent of the cultures prepared in M 150 and M 416 were found suitable for use in nutrition studies; with White's medium, approximately 50 percent of the cultures were found to be satisfactory; with Healy's 703, less than 30 percent were considered usable.

Discussion

The development of synthetic media for tissue cells requires uniform or reproducible cultures that permit quantitative evaluation of the responses obtained (*14*). In recent studies (*5,15,16*) this requirement has been met by the use of established cell strains, such as Earle's L strain (*17*) or Gey's HeLa strain (*18*). It has recently been reported (*19*), however, that two sublines derived from a single-cell clone isolated from normal mouse subcutaneous tissue differed completely in their ability to produce tumors in experimental animals. This observation raises the possibility that nutritional variants may also have arisen during the long-term cultivation of many cell strains in different laboratories. To avoid the possibility of nutritional variants in the present study, it was decided to employ freshly explanted tissues for each experiment, even though the chick embryonic-heart fragments used represented a mixed source of cell types. By careful standardization of the experimental technique, cultures were obtained that yielded relatively uniform survival times with all the media tested.

Adhesion of the chick embryonic-heart fragments to the glass surface appeared to involve the initial formation of a very thin clot of tissue exudates. Once this clot was formed, a migrating ring of fibroblasts held the fragments firmly in place. The initial clot formation was found to occur most readily under slightly acid conditions. Moistening the tissue pulp with synthetic medium M 150, prepared without bicarbonate, catalyzed the reaction, and this procedure was adopted routinely. Similar results were achieved by adding viscous materials, such as methocel or

glycerol, and surface-active agents, such as Tween 80, to the tissue pulp. However, the use of these materials represented a major alteration in technique and, in some cases, a risk of toxicity if excess materials were used.

This standardized culture technique has been used in comparative synthetic-media studies on approximately 1,000 cultures during a 2-year period. The results obtained show that the order of decreasing complexity of the synthetic media (table 1) also represents the order of the ability of these media to support cell survival (text-figs. 1 and 2). The best medium under these conditions was M 416 followed, in decreasing order, by M 150, Healy's 703, White's medium, Fischer's V-614, and Rosenberg's S-27. The low activity of Healy's 703 was somewhat surprising since it was claimed (*5*) that this medium would replace 199 for all purposes.

The ability of the media to support survival by the standardized technique reported here was found to be the same whether the cultures were first started in M 150 or directly in the different media. Exactly the same degree of usefulness was found when the cultures were evaluated microscopically after an initial 7 days in the respective media. These results, therefore, appear to reflect the nutritional adequacy of the individual media and cannot be attributed to artifacts created by the testing procedure. Since Fischer's V-614 and Rosenberg's S-27, which maintained survival for only a few days, were devised as supplements to dialyzed natural media, it would appear that large amounts of essential factors must have been supplied by the large-molecular material used. For this reason, synthetic media containing even a small proportion of such natural materials must be regarded as unsuitable for precise nutritional studies.

Since M 150 is identical in organic composition and nutritive value (*7*) with medium 199 (*4*), and since Healy's 703 (*5*) and M 416 are modifications of this same formula, it becomes possible to relate the differences in behavior of these media to specific chemical substances. Thus, the addition of certain coenzymes, plus increased levels of cysteine, ascorbic acid, and glutathione [specified for Healy's 703 (*5*)] to M 150 results in M 416 and prolonged cell survival. The beneficial effect of these substances appears clear. On the other hand, the omission of purines, pyrimidines, ATP, and adenylic acid from the composition of Healy's 703 has reduced the survival time below that of the M 150 control. It must be assumed, therefore, that the presence of these substances is essential to maintain extended survival or to promote the initial establishment of this type of tissue culture. In contrast to these observations, Healy's 703 appears to be as good as M 416 for cultivation of the L strain (unpublished data). This implies that the L strain does not have a major requirement for purines, pyrimidines, ATP, and adenylic acid. It is of interest that two recent media for the L strain (published after the completion of the present experiments) either omit these compounds entirely (*20*) or include only the single purine hypoxanthine (*21*). It must be anticipated, therefore, that removal of elementary building blocks from the present incomplete synthetic media may limit the usefulness of such media to certain cell types.

In the development of synthetic media for tissue cultures, a clear distinction must be made between media intended to stimulate rapid, short-term proliferation and media devised for long-term survival. It must also be borne in mind that media devised for one cell type will not necessarily be suitable for other cell types or other cultural conditions. The results of these experiments suggest that synthetic media can only be formulated in terms of specific cell types and precise cultural conditions and that changes in technique should be based on comparative data.

Summary

1) Improved methods have been established for the preparation of uniform cultures of freshly explanted chick embryonic-heart tissues without the use of plasma clots. Maintenance of a low *p*H or the use of viscous or surface-active agents was found to increase adhesiveness of the tissue fragments to the glass surface.

2) Synthetic media devised by various workers have been compared for their ability to maintain the survival of uniform cultures of chick embryonic-heart fibroblasts. The most suitable medium tested was found to be Morgan's M 416 followed, in decreasing order, by Morgan's M 150, Healy's 703, White's medium, Fischer's V-614, and Rosenberg's S-27.

3) The beneficial effect of coenzymes and high levels of cysteine, ascorbic acid, and glutathione in prolonging cell survival has been confirmed.

4) The essential role of purines, pyrimidines, ATP, and adenylic acid for the initial establishment and prolonged maintenance of chick embryonic-heart tissue has been demonstrated.

5) The relationship of these findings to cell nutrition is discussed.

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PLATE 128

All figures illustrate representative cultures of freshly explanted chick embryonic-heart tissue cultivated in various synthetic media. Photomicrographs prepared at the end of initial 7 days' cultivation. × 42

FIGURE 1.—Control medium M 150.

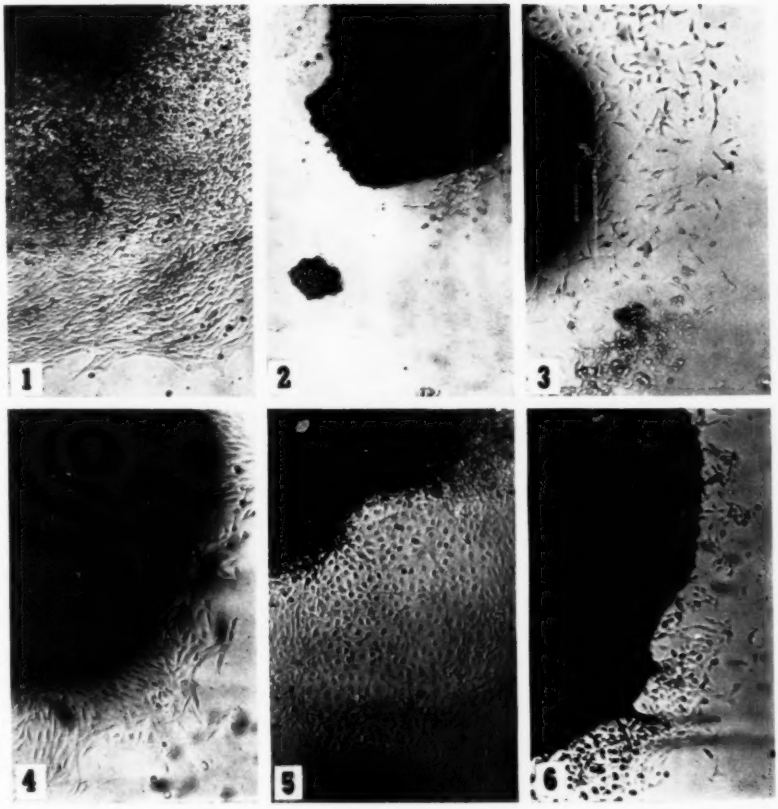
FIGURE 2.—Rosenberg and Kirk's S-27.

FIGURE 3.—Fischer's V-614.

FIGURE 4.—White's medium.

FIGURE 5.—Morgan's M 416.

FIGURE 6.—Healy's 703.



Morgan *et al.* 1415

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Smoking Patterns and Epidemiology of Lung Cancer in the United States: Are They Compatible?[1]

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Retrospective studies on patients with pulmonary cancer and matched controls have revealed uniformly an association between smoking, particularly of cigarettes, and lung cancer (*1*). Results of forward studies on British physicians (*2*) and on males interviewed by the American Cancer Society (*3,4*), in which smoking histories were collected first and the individuals then followed to determine subsequent lung-cancer-mortality experience, also show, to date, an excess risk of lung cancer among cigarette smokers.

It must be accepted that an association between smoking and an increased risk of lung cancer has been observed repeatedly. The inference from this association of a causal relationship, however, has been challenged. The following points of criticism have been stressed: *a*) the groups studied were selected and not representative cross sections of the population, so that the findings cannot be generalized to the total population, and *b*) smokers constitute a self-selected group, and the decision to smoke or to abstain may be influenced by other characteristics, so that the association between smoking and lung cancer may merely reflect a further association of lung cancer with other unidentified causative factors.

The importance of the problem of pulmonary cancer, and of the inferences to be drawn from the reported data, make additional means of testing the evidence very desirable. The potential applicability of findings from studies on restricted and selected groups to the total population is particularly significant from the public health standpoint.

The statistical investigation we are reporting was designed to test whether the results of special retrospective and prospective studies in the medical literature were compatible with more broadly based information on the distribution of lung cancer, and thus presumably valid for generalization to larger populations. The material presented here is obviously not intended as a *de novo* attempt to establish or to refute an excess lung-cancer risk among cigarette smokers nor to clarify the exact nature of this association.

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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Materials and Methods

For present purposes information on the distribution of lung cancer and on the smoking habits in the total population is necessary.

Data on lung cancer and on smoking.—Data on the epidemiologic characteristics of lung cancer in the United States are available from several independent sources. Four were selected for this study: *a*) mortality data published by the National Office of Vital Statistics and also additional detailed tabulations by the same office which have been analyzed by Hoffman and Gilliam (*5*); *b*) the incidence data of 1947–51 from the Connecticut cancer register (*6*); *c*) the 10-city survey conducted by the National Cancer Institute in 1948, which yielded prevalence, incidence, and mortality data (*7*); and *d*) the Iowa survey of 1950, which supplemented the 10-city data with figures on urban-rural differences (*8*).

The consistency of these data could be checked by a number of internal analyses. One important and gratifying feature was that, for both the 10-city survey and the Iowa survey, the relative differentials by sex, age, and several other characteristics for a variety of sites, including lung and bronchus, agreed very well when determined from total mortality figures or from data restricted to newly diagnosed, microscopically proved cases. These analyses convinced us that mortality data may be more adequate for the purpose of comparing population groups than is commonly believed. Because of the inaccuracies inherent in general statistics on cancer mortality and morbidity, no dependence was placed on estimates of the absolute magnitudes of the rates, and the investigation was limited to a study of relative differences among major population groupings. The basic data on the incidence and mortality from lung cancer in the United States used in this investigation are summarized in tables 1, 6, 9, 12, and 15.

Data on the distribution of the smoking patterns in the United States population were not available. The few studies that have been published covered only restricted areas or groups and could not be considered as representative of the total population. This was one of the reasons that led the National Cancer Institute to contract with the Bureau of the Census for the collection of smoking histories on a representative cross section of the United States population comprising 45,000 persons 18 years of age and over. The questions on smoking habits in terms of type and intensity of use were patterned after those used in the American Cancer Society (*3*) and Veterans Administration-National Cancer Institute studies. They were designed to classify individuals and not to estimate total current consumption. However, the estimate of current cigarette consumption which could be derived from the survey data was only 15 percent under the total national aggregate as determined from tax-revenue figures. This underestimate of a national aggregate is a rather general property of survey data and has been noted in surveys for items other than tobacco. The relative differences in smoking habits among population groups found here are generally consistent with the picture which can be pieced together from other studies and with impres-

sions of trade sources. The detailed findings are published elsewhere (*9*) and cover the variation of smoking habits by sex, age, color, residence, and other parameters. Tables 2, 7, 10, 13, and 16 summarize some results on the distribution of cigarette-smoking patterns, since these findings are of primary concern in the present investigation.

Statistical method.—The relationships between these two sets of data can be investigated by direct or indirect methods.

A direct approach would be to classify all lung-cancer deaths according to smoking history, to observe whether lung-cancer gradients by intensity of tobacco use would persist within a variety of groups categorized by age, sex, place of residence, and other characteristics. To handle the smoker self-selection issue, the total lung-cancer rate for each population component, including all classes of smokers and nonsmokers, would also have to be examined. The feasibility of collecting smoking histories on a random sample of all deaths from lung cancer registered in the United States is certainly worth exploring. Such a study would also have to ascertain the reliability of smoking histories obtained from relatives of the deceased.

An indirect method that is also applicable to the analysis is analogous to the often-employed "indirect" procedure for age-standardizing rates. It consists of adopting a schedule of rates (or relative risks) for nonsmokers and smokers, graded according to amount used (smoking class), from some known experience and applying it to the population to obtain "expected" values for comparison with the observed values. For lung cancer the relative risks by smoking class may be derived from the results of the retrospective and prospective studies. In effect, the model of rates derived from special studies permits a test of the consistency between independent information on the distribution of lung cancer and on the distribution of smoking habits. It might be noted that such rates were published long before the present investigation was instituted or, for that matter, before adequate data on the smoking patterns in the population were gathered.

The indirect method of analysis achieves results comparable with the direct method for estimation of how much of the observed variations in lung-cancer epidemiology can be accounted for if smoking habits are controlled statistically. Stocks (*10*) recently used this approach in England by applying the mortality risks as estimated by Doll and Hill to distributions of smoking histories for selected population samples.

Model adopted for test.—Since lung cancer occurs among nonsmokers, the risk of lung cancer among nonsmokers will be expressed as a unit risk, and the excess risk for various smoking classes, as multiples of the unit risk. Age is controlled in all comparisons, and the model assumes that the statement of *relative* risks applies within each age group.

The retrospective and prospective studies on white males have shown differences of the order of 7–8 to 1 between cigarette smokers and nonsmokers, and for smokers of over a pack daily the ratio rises to as much as 13–15 to 1. The schedule of relative risks of lung cancer among male

cigarette smokers and nonsmokers was adapted from the analysis of the three largest retrospective studies as published by Cutler and Loveland (*11*), and from data reported by Hammond and Horn (*4*) in their prospective study. In the retrospective studies, cigar and pipe smoking were converted into cigarette equivalents and published data were not available for cigarette smokers only. Since available evidence reports that cigar and pipe smokers do not have as high a lung-cancer risk as cigarette smokers, the retrospective results should understate to a small degree the spread of relative risks which would be found for cigarette smokers only. There is, in any event, close numerical agreement between these two sets of results:

	Retrospective (Cutler-Loveland)	*Prospective (Hammond-Horn)*
Nonsmokers	1	1
½ pack or less daily	4	6½
½ to 1 pack daily	8	6½
More than 1 pack daily	13	14

While the Hammond-Horn figures show no progression in risk from the ½-pack or less to the ½- to 1-pack category, the average value for the two classes combined agrees almost exactly with that derived from the retrospective studies. The Hammond-Horn figures for ½ pack or less may reflect sampling variation or be temporarily influenced by the inclusion of persons who recently reduced their amount of smoking. Prolonged observation may establish a pattern of orderly progression of risk which has been observed in the retrospective studies.

The consistency between retrospective and forward studies suggests that additional retrospective studies and the extension of the follow-up period in forward studies would not materially alter the statements of relative risk obtained from these sources. Berkson (*12*), who has raised the selection issue in connection with forward studies, agrees that continued differences between smokers and nonsmokers after an initial 3-year observation period would force recasting of his criticism.

The relative risks are taken as applying to current cigarette-smoking rates unless noted to the contrary, although examination of the smoking data for the population indicates that "current" and "maximum" rates would yield substantially the same comparative results within each age group. Cigar and pipe smoking have not been taken into account here since these forms are not so commonly used and have not been associated with lung cancer to nearly the same degree as cigarettes.

A statement of relative risk for discontinued regular cigarette smokers is included, even though the rather consistent proportion of discontinued smokers in a variety of groups shows that this is not a determining factor in the results which follow. Some preliminary data of Hammond and Horn suggest 3 to 1 as an appropriate measure of risk. It might be noted parenthetically that this ratio would not necessarily imply that discontinuance reduces the lung-cancer risk, and this latter point would require further observation and study, including analysis of the mortality experience for all other causes among discontinued smokers.

The model of relative risks adopted for this study is, therefore, as follows:

Nonsmokers of cigarettes	1
Discontinued regular cigarette smokers	3
Regular cigarette smokers:	
½ pack or less daily	4
½ to 1 pack daily	8
More than 1 pack daily	13

In the subsequent sections, this model of relative risks is applied to the analysis of the sex ratio, the urban-rural ratio, the white-nonwhite ratio, to regional variation, and to relationships between groups born during different time periods, as well as to some secondary differences within the main variables. The question being investigated may be restated as follows: If the relative risks of lung cancer among smokers and nonsmokers, as derived from special epidemiologic studies on selected groups of men, are systematically applied to components of the total population, how do the resultant derived or "predicted" figures compare with the lung-cancer morbidity or mortality relationships observed for the same population groupings?

Sex Ratio

The very high male-female ratio of mortality rates is a prominent characteristic of lung cancer. In this respect lung cancer now outranks all other leading causes of death, including syphilis and tuberculosis. Table 1 summarizes some available data on sex ratios of lung-cancer incidence and mortality rates. The ratios vary with the source of the material and have risen in recent years. The most recent sex ratio for the United States (1953) (*13*) is of the order of 5 to 1. Lung-cancer deaths are concentrated in the ages over 45 years, and the relative age profiles of the rates are similar for both sexes between the ages of 45 and 75, so that the choice of a rate (crude or age-adjusted) for determining the sex ratio makes little difference. The sex ratio should be less subject to variation in diagnosis and case finding than comparisons based on race, place of residence, or region.

Since few lung-cancer deaths occur among persons under 35, only smoking patterns for persons over that age would be relevant to a discussion of past or current lung-cancer morbidity and mortality. Table 2 abstracts the information on cigarette-smoking patterns by age and sex.

The schedule of relative risk stated in the model was applied to the percentage distribution by cigarette-smoking class for each age-sex group in table 2 to obtain a composite estimate of risk for each group. It should be emphasized that the application of the schedule of relative risks to women, established by observation among men, is an assumption made deliberately to see how many of the epidemiologic characteristics of lung cancer among women might be accounted for by this particular model. The fragmentary published data on smoking histories among women lung-cancer patients and controls (*14–16*) suggest a gradient in

TABLE 1.—*Reported lung-cancer incidence and mortality rates per 100,000 population by sex, with sex ratios**

	Crude rate						Adjusted rate (all ages)		
	All ages			35 years and over					
	Male	Female	M/F	Male	Female	M/F	Male	Female	M/F
Incidence									
Connecticut register, 1947–51 (*6*)	28. 3	5. 2	5. 4	62. 1	10. 9	5. 7	26. 8	4. 6	5. 8
Ten-city survey, 1948 (*7*)	28. 9	6. 4	4. 5	63. 9	13. 8	4. 6	29. 2	6. 5	4. 5
Iowa survey, 1950 (*8*)	21. 1	7. 9	2. 7	47. 4	16. 8	2. 8	18. 8	6. 6	2. 8
Mortality									
United States, 1948–49 (*7*)	17. 7	4. 4	4. 0	41. 2	9. 9	4. 2	17. 8	4. 3	4. 1
United States, 1953 (*13*)	25. 6	5. 1	5. 0	60. 5	11. 4	5. 3	26. 1	4. 9	5. 3

**Note:* Where indicated, rates adjusted to the age distribution of the United States 1950 census population.

TABLE 2.—*Percent distribution of population with respect to current cigarette-smoking patterns, by sex and age**

Sex and age	Total	Current daily cigarette-consumption rate				
		Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10 to 20 cigarettes	Over 20 cigarettes
Males						
35–44 years	100.0	26.7	11.1	8.4	34.9	18.9
45–54 years	100.0	30.9	12.2	8.7	31.6	16.6
55–64 years	100.0	41.1	15.3	9.1	23.9	10.6
65 years and over	100.0	65.1	12.7	7.9	10.8	3.5
Females						
35–44 years	100.0	63.0	4.6	10.9	18.3	3.2
45–54 years	100.0	73.9	3.3	7.9	12.0	2.9
55–64 years	100.0	86.9	2.2	4.7	5.1	1.1
65 years and over	100.0	95.2	1.2	1.9	1.5	0.2

**Note:* The small number of persons in the sample for whom smoking histories were not obtained have been distributed proportionately.

Data abstracted from Current Population Survey for February 1955 (*9*).

lung-cancer risk between women smokers and nonsmokers, but they do not suffice for numerical estimates. The ratios of the composite risks are set forth in the first column of table 3. The over-all ratio for 35 years and over is based on weighted sums of the composite risks, the weights representing the proportion of lung-cancer deaths in each age group.

The distribution of smoking histories is based on a total sample of about 45,000 persons 18 years of age and over and, hence, is subject to sampling error. The standard errors of the ratios predicted by the model due to sampling error in the estimated distribution of smoking habits have been indicated in table 3 and its succeeding counterparts. The standard errors of the predicted sex ratios are not large enough to modify the interpretation of the results in tables 1 and 3. The sampling errors of the observed sex ratios in table 1 are trivial compared to other possible sources of reporting error and have not been given.

The male-female ratio predicted for all ages over 35, 2.4 to 1, is roughly half of the observed ratio of the order of 5 to 1. However, substantial sex

TABLE 3.—*Male-female ratios (M/F) of lung-cancer rates predicted by model*

Age (*year of birth*)	Without adjustment for years of exposure to cigarette smoking	With adjustment for years of exposure to cigarette smoking
35 years and over (before 1920)	2.4 ± 0.05	3.6 ± 0.07
35–44 years (1910–19)	2.0 ± .04	2.4 ± .05
45–54 years (1900–09)	2.3 ± .06	3.1 ± .08
55–64 years (1890–99)	2.7 ± .09	4.2 ± .14
65 years and over (before 1890)	2.2 ± .08	3.5 ± .12

differences exist in the ages at which smoking was started, so that duration of exposure has not been the same for the two sexes. Table 4 gives the median starting ages of smoking for men and women.

TABLE 4.—*Median starting age for males and females taking up regular cigarette smoking**

Age as of February 1955	Males	Females
35–44 years	18.2	21.3
45–54 years	18.4	26.0
55–64 years	18.6	35.3
65 years and over	19.3	39.9

**Note:* Data abstracted from Current Population Survey for February 1955 (*9*).

Since the smoking-survey data reveal that persons maintain about the same smoking rates over long time periods, differences in length of exposure imply variation, as well, in total dose of any active agents in cigarette smoke. The lung-cancer risks by cigarette-smoking rate found in studies on men suggest a roughly linear relationship between risk and intensity of use (and total dose); such observations could fit into the lower range of a dose-response curve, where total dose is represented on normal or log-normal scale, bearing in mind that cigarette smoke must be at best a weak carcinogen. Experimental work on several agents has resulted in conventional dose-response-curve relationships between log total dose and tumor response (*17*). If total dose is the important determinant in lung-cancer response, the model here being investigated should be modified by weighting the results on relative risks for each group, which are based on rate of use, by the average number of years of exposure. For this situation, the risk becomes proportional to years of exposure. When duration of exposure is the same for two groups being contrasted, the correction has no effect.

This correction probably oversimplifies the true situation. Cramer and Stowell (*18*), working on the induction of skin tumors with methylcholanthrene in Swiss mice, have reported a duration-of-application effect, the total dose necessary to induce 100 percent response diminishing as applications became increasingly discontinuous. This suggests that an increase in response rate should be more than directly proportional to an increase in exposure time. Few other experimental data are available which measure the effect of varying duration of exposure. Epidemiologic data on occupational cancers report only length of exposure of known cases. However, taking advantage of the property that the population exposed to risk decreases monotonically with increased duration of exposure, the data on scrotal cancer among mule spinners and bladder cancer among aniline workers (*19*), for example, indicate that risk rises at a rate probably greater than in direct proportion to length of exposure.

The exact nature of the effect of duration of smoking on lung-cancer risk can ultimately be worked out from studies in progress. In the absence of data it might be pointed out that many theoretical distributions of

cases with respect to time (including the normal and rectangular distributions) lead to incidence rates with an increase greater than direct proportionality, but in the low dosage-low response range the departure from linearity would not be too great. For present purposes the assumption that lung-cancer risk is directly proportional to years of exposure, as measured from age at which regular cigarette smoking started, seems conservative and has been used for adjusting the expected sex ratios, the results of which appear in the second column of table 3.

The modified sex ratio predicted for all ages over 35 is 3.6 to 1. Comparison of the observed 5 to 1 ratio with this predicted ratio implies there is a male excess in lung-cancer mortality of about two fifths over and above the female rate that cannot be accounted for by standardization for smoking history. This residual male excess does not differ greatly from sex ratios observed for mortality from other causes at ages over 35. For example, in 1950 the male-female ratios of mortality rates among persons 35 years and over were as follows: all causes, 1.43 : 1; pneumonia and influenza, 1.44 : 1; cancer of sites not involving reproductive organs, exclusive of respiratory system, 1.42 : 1. One may conclude that adjustment for cigarette-smoking history tends to bring the sex ratio for lung-cancer mortality into much closer conformity with those observed for other causes of death.

There is corroborative detail which may be mentioned. Because of the timing in the adoption of the cigarette habit the model calls for the largest sex difference to be found among persons 55 to 64 years, those born between 1890 and 1900. The smoking-survey data show (see text-fig. 2) that the habit became popular among males around the time of World War I, while its adoption by women came later; sex differences in the proportion of cigarette smokers have narrowed in the younger cohorts. Observations from the 1950 and 1953 mortality data conform to this prediction. Table 5 shows further that the maximum sex ratio has not always been fixed at 55 to 64 years, but rather indicates a trend toward an older age for the maximum sex ratio, which suggests that the maximum sex ratio is a cohort characteristic rather than a property of some fixed point in the life span. This cohort property would be consistent with the model. The age pattern of observed sex ratios has been influenced by the greater stability of the female lung-cancer rates since 1935 for persons under 65 years. This is discussed further in a later section on cohort comparisons.

TABLE 5.—*Sex ratios of age-specific lung-cancer-mortality rates. United States: 1930, 1935, 1940, 1945, 1950, and 1953*

Age	1930	1935	1940	1945	1950	1953
35–44 years	1. 9	2. 2	3. 1	2. 7	3. 6	3. 5
45–54 years	2. 3	2. 6	3. 5	3. 9	5. 7	6. 3
55–64 years	1. 6	2. 2	3. 3	3. 9	5. 9	7. 7
65–74 years	1. 5	1. 9	2. 4	2. 8	4. 0	5. 3
75–84 years	1. 3	1. 6	2. 3	2. 0	2. 4	3. 1

Urban-Rural Ratio

Historically, the death rate from all causes has always been greater in urban than in rural populations and this relationship carries over to urban-rural comparisons of deaths from all malignant neoplasms. Within this latter category, however, the urban-rural ratios of mortality rates for lung and bronchus, and buccal cavity as well, are substantially larger than those noted for other primary sites.

In contrasting urban and rural experience for any single cause of death, at least three factors may operate to inflate urban-rural ratios: *a*) overstatement of urban residence on death certificates due to failure to distinguish between place of residence and post-office address, *b*) better diagnostic facilities in urban areas, and *c*) a possible tendency of sick people to migrate to urban centers for medical care. However, there seems to be an urban-rural effect not specific for lung cancer or any single cause. At ages 45 and over, the urban-rural ratios for mortality from all causes range from 1.10 to 1.35 and persist on a smaller scale in comparisons of metropolitan and nonmetropolitan counties. It is hard to visualize how such a large urban excess as this could be produced by wholly artificial classification means.

The overstatement of urban residence on birth certificates has been investigated (*20*). Failure to differentiate between place of residence and post-office address usually occurs only in reporting events in hospitals and must be a more important source of error for birth certificates than for lung-cancer deaths. Most births now occur in hospitals, while little more than half of these deaths take place away from home, according to a tabulation made by the National Office of Vital Statistics. Applying the birth-certificate results to deaths in institutions one can reasonably estimate as much as a 3 percent overstatement in the urban lung-cancer rate nationally and a 5 percent understatement in the rural rate due to incorrect residence information supplied on the death certificate. To adjust for this one factor the observed urban-rural lung-cancer ratios might be reduced by as much as 5 to 10 percent.

Table 6 presents age-adjusted data on lung-cancer incidence and mortality for urban and rural areas. All sources agree that urban-rural ratios for males exceed those for females and the national data for 1948–49 may be accepted as typical. The term "rural" embraces both farm and nonfarm populations and in some areas the latter probably resembles the urban population. Urban-rural comparisons do not have the same meaning everywhere because of sectional differences in the proportion of farm and nonfarm populations. This factor probably accounts for much of the difference between the Connecticut and Iowa observations. From analysis of the 1948–49 lung-cancer mortality data presented by Hoffman and Gilliam (*5*) it can be shown that the Northeastern states, including Connecticut, with sizable nonfarm populations have lower urban-rural ratios than other parts of the country where the farm population is proportionately greater.

Table 7 summarizes the distribution of cigarette-smoking histories for

TABLE 6.—*Age-adjusted lung-cancer incidence and mortality rates per 100,000 population, by sex and urban-rural residence, with urban-rural ratios**

	Male			Female		
	Urban	Rural	U/R	Urban	Rural	U/R
Incidence						
Iowa, 1950 (*8*)	29. 0	10. 2	2. 84	7. 8	5. 3	1. 47
Connecticut, 1947–51 (*6*)	27. 9	17. 8	1. 57	4. 7	3. 8	1. 24
Mortality						
United States, 1948–49 (*5*)	22. 0	11. 9	1. 85	4. 7	3. 6	1. 31
35–44 years	8. 0	4. 9	1. 63	2. 4	1. 5	1. 60
45–54 years	38. 3	22. 0	1. 74	7. 0	5. 0	1. 40
55–64 years	91. 2	48. 9	1. 87	14. 7	11. 2	1. 31
65 years and over	100. 4	51. 3	1. 96	26. 5	21. 0	1. 26

**Note:* Rates adjusted to the age distribution of the United States 1950 census population.

the urban, rural nonfarm, and farm populations. There is close correspondence in the urban and nonfarm results, particularly for males, the sharp break appearing between the nonfarm and farm populations. Unfortunately, the present lack of separate morbidity and mortality data for nonfarm and farm populations forces their consolidation into an over-all rural category. Urban-rural differences in age at which smoking was started are small and need not be considered here.

The procedure described in the preceding section on sex ratios was followed for deriving the predicted urban-rural ratios given in table 8. For all ages over 35 years the schedule of relative risks forecasts a 13 and 15 percent excess, respectively, among urban males and females. For women the correspondence between prediction and observed facts is not too bad when the possibilities for inflation of the observed urban-rural ratio are recalled. The result does not force one to discard the possibility of accounting for nearly all the urban-rural differences in that sex through the standardization of smoking histories, although the need for postulating the operation of some other factor(s) is suggested. For men the prediction from the model falls so far short of the urban-rural difference observed that one must conclude that standardization for smoking history cannot conceivably account for all of it.

This conclusion does not seem inconsistent with the findings in two of the special studies among men. Doll and Hill (*14*) and Hammond and Horn (*4*) both commented that differences in risk between urban and rural populations in their studies could not be attributed wholly to differences in smoking habits. Stocks and Campbell in their preliminary findings, based on a follow-up of all reported lung-cancer deaths occurring in Liverpool, England, and some adjacent areas, have reported substantial differences in lung-cancer mortality between urban and rural males when smoking rate is held constant (*21*); their data suggest that the relative urban excess is greater for nonsmokers and light cigarette smokers than for moderate and heavy cigarette smokers. The size of the male urban-rural difference not accounted for by indirect adjustment of smoking history does not appear to support some computations by Doll which

TABLE 7.—*Percentage distribution of population with respect to current cigarette-smoking patterns, by sex, age, and urban-rural residence**

Age and residence	Current daily cigarette-consumption rate											
	Males						Females					
	Total	Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10–20 cigarettes	Over 20 cigarettes	Total	Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10–20 cigarettes	Over 20 cigarettes
Urban												
35–44 years	100.0	25.1	10.5	8.2	35.8	20.4	100.0	59.0	4.7	11.9	20.7	3.7
45–54 years	100.0	30.3	11.5	9.0	31.3	17.9	100.0	71.0	3.4	9.1	13.2	3.3
55–64 years	100.0	38.4	16.2	8.9	25.1	11.4	100.0	85.4	2.3	5.6	5.5	1.2
65 years and over	100.0	62.4	13.7	8.6	11.1	4.2	100.0	94.5	1.5	2.1	1.8	0.1
Rural nonfarm												
35–44 years	100.0	27.3	13.4	7.7	34.4	17.2	100.0	64.1	4.9	11.5	17.3	2.2
45–54 years	100.0	28.0	13.7	7.3	35.8	15.2	100.0	75.9	3.5	5.6	12.3	2.7
55–64 years	100.0	39.6	14.6	10.7	23.4	11.7	100.0	87.4	2.1	4.0	5.2	1.3
65 years and over	100.0	65.4	11.4	7.2	13.2	2.8	100.0	95.9	0.9	1.8	1.1	0.3
Rural farm												
35–44 years	100.0	32.7	11.0	11.0	32.1	13.2	100.0	85.8	3.4	4.0	5.9	0.9
45–54 years	100.0	37.9	13.1	9.4	27.2	12.4	100.0	89.5	1.8	3.8	4.4	0.5
55–64 years	100.0	54.8	12.0	8.2	19.0	6.0	100.0	95.3	1.3	0.9	2.0	0.5
65 years and over	100.0	73.4	11.1	6.3	7.2	2.0	100.0	98.4	0.6	0.6	0.4	—

**Note:* Data abstracted from Current Population Survey for February 1955(*9*).

TABLE 8.—*Urban-rural ratios (U/R) of lung-cancer rates predicted by model*

Age (*year of birth*)	Male	Female
35 years and over (before 1920)	1. 13±0. 04	1. 15±0. 03
35–44 years (1910–19)	1. 11± . 03	1. 34± . 05
45–54 years (1900–09)	1. 07± . 04	1. 28± . 06
55–64 years (1890–99)	1. 13± . 06	1. 16± . 06
65 years and over (before 1890)	1. 15± . 07	1. 08± . 04

led him to speculate that lung-cancer incidence among nonsmokers might be about the same ". . . in residents in areas of different density of population . . . " (*22*).

Although smoking histories clearly account for only part of the urban-rural differences in the model, it should be emphasized that the result is not inconsistent with the presence of excess risks for smokers as indicated by retrospective and forward studies of selected populations. The Stocks-Campbell data, which show marked gradients by smoking class for urban and rural males separately, support this view.

It is interesting that the model correctly forecast (table 8) divergent age patterns of the urban-rural ratios for each sex. Among women the observed ratios become smaller with advancing age as predicted. The prediction for men was no change or possibly a small rise in the ratio with age, and a rise with age is observed.

The arithmetic of the present comparisons suggests that the model postulating equal risks for male nonsmokers and smokers in urban and rural environments must be modified. To investigate this point, supplemental data for lung-cancer deaths in selected parts of the United States would prove useful. Comparison of mortality among urban and rural nonsmokers would be one direct measure of differences assignable to urban environmental factors, and the interaction of these factors with smoking could be covered by urban-rural comparisons for specific cigarette-smoking classes.

The possible better fit of the observed female urban-rural differentials to the model raises some interesting questions. If American experience should reveal that the smallest urban-rural differences occur among nonsmokers, the sex difference in testing the model against the observed facts might be explained, since there are many more women nonsmokers. The British data, which Stocks and Campbell feel may incriminate total intake of benzo[*a*]pyrene through cigarette and air pollution, would not lead one to expect such a finding. On the other hand, if the lower urban-rural ratio for women could be traced to smaller urban-rural differentials for female nonsmokers, this would call for close scrutiny of suspected agents in the urban environment to see if sex differences in exposure to these agents could be demonstrated.

Because of the great differences in smoking patterns between the nonfarm and farm populations, along with the promise of removing some of the complicating urban environmental factors from the analysis, it would be interesting to ascertain if a nonfarm-farm lung-cancer differen-

tial exists and if so, how much of it could be accounted for by standardization of smoking history. A question on farm residence will be added to the death certificates in many states and data on this point should be available after 1958. Among males a partial answer may be supplied by examination of the 1950 occupational mortality data for farmers when these become available.

White-Nonwhite Ratio

Although nonwhites have a higher over-all death rate than whites, the situation is reversed for such causes of death as cardiovascular diseases and malignant neoplasms. This race relationship is highly variable for individual cancer sites, the relatively small higher white incidence rate for lung and bronchus occupying a position intermediate to the huge white excess in skin cancer and the larger nonwhite rate for cervix and stomach.

Comparison of white and nonwhite age-specific mortality rates at older ages is rendered difficult because of inaccuracies in statements of age for the nonwhite population, a problem well known to actuaries preparing life tables for nonwhites (*23*). For this reason the data on lung and bronchus in table 9 have been restricted to summary age-adjusted rates. The available information on white-nonwhite differentials comes from the 10-city morbidity survey and United States mortality data. The 10-city cancer-incidence data are probably the more accurate for males, in which nonwhite cases are represented in adequate numbers, since differences in diagnostic and case-finding facilities associated with race should be minimal in the large urban medical centers. Since 1950, the United States mortality data have exhibited a trend to smaller white-nonwhite ratios for lung cancer.

TABLE 9.—*Age-adjusted lung-cancer incidence and mortality rates per 100,000 population by sex and color, with white-nonwhite ratio**

	Male			Female		
	White	Non-white	W/NW	White	Non-white	W/NW
Incidence						
Ten-city survey, 1948 (*7*)	29.5	25.4	1.16	6.5	5.8	1.12
Mortality						
United States, 1948–49 (*5*)	18.3	13.1	1.40	4.4	3.4	1.29
Urban	22.3	16.9	1.32	4.7	4.2	1.12
Rural	12.3	7.3	1.68	3.7	2.1	1.76

**Note:* Rates adjusted to age distribution of the United States 1950 census population.

Because of the relatively small number of nonwhites sampled, the survey data on cigarette-smoking patterns have been pooled in table 10 for the population 45 years and over. The survey indicates not much difference between whites and nonwhites in the total proportion of cigarette smokers. However, whites have a greater proportion of heavy smokers using more than one pack of cigarettes daily. A similar finding has turned up in one other study (*24*). An interesting race distinction is

TABLE 10.—*Percent distribution of population 45 years of age and over with respect to current cigarette-smoking patterns, by sex, color, and urban-rural residence**

Color and residence	Current daily cigarette-consumption rate											
	Males						Females					
	Total	Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10 to 20 cigarettes	Over 20 cigarettes	Total	Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10 to 20 cigarettes	Over 20 cigarettes
White	100.0	43.0	13.8	8.1	23.5	11.6	100.0	84.1	2.4	5.0	6.9	1.6
Urban	100.0	41.4	14.0	8.1	23.9	12.6	100.0	82.1	2.5	5.9	7.6	1.9
Rural nonfarm	100.0	41.3	13.7	8.2	25.9	10.9	100.0	85.3	2.4	3.7	7.0	1.6
Rural farm	100.0	54.4	12.5	7.4	18.3	7.4	100.0	94.1	1.4	1.5	2.6	0.4
Nonwhite	100.0	48.3	8.6	15.3	22.3	5.5	100.0	86.1	1.7	6.3	5.2	0.7
Urban	100.0	43.2	8.7	16.3	24.9	6.9	100.0	84.6	2.3	6.5	5.7	0.9
Rural nonfarm	100.0	62.8	8.6	10.0	17.0	1.6	100.0	89.5	—	6.9	3.6	—
Rural farm	100.0	60.3	7.3	15.2	13.9	3.3	100.0	91.6	—	5.6	2.8	—

*Note: Data abstracted from Current Population Survey for February 1955 (9).

also found in the nonfarm population, the smoking pattern for whites resembling that of urban whites and the pattern for nonwhites resembling that of farm nonwhites. The distribution of occupations in the nonfarm population suggests that nonwhites are less exposed to urban influences.

The differences in relative risks between white and nonwhite predicted by the model in table 11 are rather small and can be attributed to the two points just noted about the distribution of smoking histories. The predictions agree well with the observations from the 10-city survey, but fall below those reported by the mortality data. The latter is not surprising since some inflation of the white-nonwhite mortality ratio due to differences associated with diagnostic facilities might be expected. Because of the marked disparity noted in smoking histories for the nonfarm population, the model calls for a substantially larger white-nonwhite ratio among rural males and this feature does appear in the United States mortality data. The meaning to be attached to this latter result is questionable, since a high white-nonwhite ratio, not predicted by the distribution of smoking histories, is also observed among rural women.

TABLE 11.—*White-nonwhite ratio (W/NW) of lung-cancer rates predicted by model*

Residence	Male	Female
Total	1.18 ± 0.06	1.14 ± 0.05
Urban	1.11 ± .06	1.14 ± .06
Rural	1.45 ± .14	1.15 ± .09

Regional Variation

Most interstate comparisons of cancer mortality have little utility because they have not been standardized for age. For lung and bronchus Hoffman and Gilliam have published 1948 and 1949 mortality data by states, which have been adjusted for age and other variables (*5*). The population surveyed on smoking habits was large enough to permit estimates for the four major regions of the country. Standardized lung-cancer-mortality ratios (1948–49) have been presented in table 12 for the same regional grouping of states. Because of the small number of deaths in each region the sampling variation of the observed mortality ratios must be considered, and the standard errors are presented. The rural areas show more regional variation for males. However, in the absence of separate mortality data for the nonfarm and farm populations, a composite rural experience had to be used by Hoffman and Gilliam in standardizing their data. The reported lung-cancer-mortality experience for the male nonfarm population is almost certainly higher than for the male farm population, and the higher rural standardized mortality ratios (SMR) for Northeastern and Western states reflect the higher nonfarm proportion in their rural populations. The percentages of nonfarm persons in the rural population as of the 1950 census were as follows: Northeast, 77.9; North Central, 53.4; South, 50.9; West, 67.4. Because of the difficulty in handling the rural data, the regional comparisons are

TABLE 12.—*Standardized lung-cancer-mortality ratios (SMR), with standard errors, for geographic regions by sex and urban-rural residence. United States, 1948 and 1949**

Residence	United States	Northeast	North Central	South	West
			Males		
Total	100	118 ± 1.1	90 ± 1.1	92 ± 1.3	98 ± 1.7
Urban	100	115 ± 1.2	91 ± 1.3	91 ± 1.7	94 ± 1.9
Rural	100	133 ± 2.8	87 ± 2.0	92 ± 2.0	108 ± 3.3
			Females		
Total	100	109 ± 2.2	96 ± 2.2	95 ± 2.5	98 ± 3.3
Urban	100	110 ± 2.4	95 ± 2.6	91 ± 3.3	101 ± 3.9
Rural	100	105 ± 5.2	100 ± 4.0	100 ± 3.8	90 ± 6.5

**Note:* The standardized mortality ratios for regions express the regional rate as a percentage of the total U. S. rate, after adjustments by the indirect method for age and race, and also urban-rural residence when indicated, have been made.

The results presented are primarily a rearrangement of data published in (*5*). We are indebted to Dr. A. G. Gilliam for making available unpublished cross-tabulations by sex and urban-rural residence.

best restricted to the urban population. This approach also has the added advantage of probably minimizing the effect of diagnosis on regional variation.

When the sampling variation of the observed urban male SMR's for each region are taken into account one cannot reject the hypothesis that the lung-cancer experience is substantially the same for the North Central, Southern, and Western states and any variation among them seems likely to be small. Males in the Northeastern states have a higher SMR than in the three other regions. Among urban women the Northeast SMR seems significantly higher than the North Central and Southern ratios, and the difference between the Western and Southern ratios is of borderline significance.

The distribution of cigarette-smoking patterns for the urban population 45 years of age and over in the four regions is set forth in table 13. The regional indices of lung-cancer mortality predicted by the model are in table 14; because of the small samples on which the regional distributions of smoking histories are based, the standard errors of the predicted ratios are rather large and the tests of the model against the observed regional indices are at best suggestive. Within limits imposed by the size of the sample the model says that for males little regional variation in the SMR's should be expected. The Northeast SMR does not conform, but the results for the other three regions are not inconsistent with this statement. The model for females calls for the SMR's for Northeastern and Western regions to be higher than for the other two regions, and this feature does appear.

The high lung-cancer rates in New York City and adjacent cities in the immediate metropolitan area contribute to the high Northeast SMR's in table 12. Excluding these cities brought the Northeastern female SMR to the level of the Western result but still left the male SMR significantly higher than for the other regions. The question remains open as to whether environmental, occupational, or diagnostic factors

TABLE 13.—*Percentage distribution of urban population 45 years of age and over with respect to current cigarette-smoking patterns, by sex and region**

Sex and region	Current daily cigarette-consumption rate					
	Total	Never smoked cigarettes regularly	Smoked cigarettes regularly but not at present	Less than 10 cigarettes	10 to 20 cigarettes	Over 20 cigarettes
Males						
Northeast	100.0	44.2	13.5	8.2	20.1	14.0
North Central	100.0	43.0	12.1	9.2	25.1	10.6
South	100.0	39.7	13.2	9.3	26.1	11.7
West	100.0	35.3	17.0	9.2	27.0	11.5
Females						
Northeast	100.0	81.0	3.0	6.4	6.9	2.7
North Central	100.0	84.4	1.3	6.0	7.2	1.1
South	100.0	83.9	3.2	5.1	6.6	1.2
West	100.0	79.2	2.4	6.6	10.7	1.1

**Note:* Data abstracted from Current Population Survey for February 1955 (*9*).

TABLE 14.—*Standardized urban lung-cancer-mortality ratios (SMR) predicted by model*

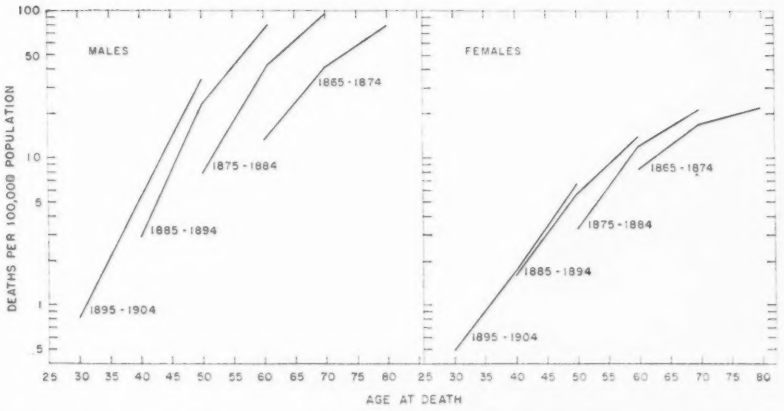
Region	Male	Female
United States	100	100
Northeastern	99 ± 2.1	105 ± 2.8
North Central	98 ± 2.1	94 ± 2.6
South	102 ± 2.6	93 ± 3.0
West	105 ± 2.9	109 ± 4.3

are operating in the urban areas of Northeastern States to a stronger degree than elsewhere in the country.

Cohort Relationships

Text-figure 1, which covers the period from 1930 to 1950, illustrates a relationship pointed out by Korteweg and others (*25, 26*), that groups of persons (cohorts) born during successive decades, dating back at least to 1875–84, have continued to experience greater lung-cancer mortality at comparable ages than their immediate predecessors. This shift in rates by cohorts has been much more prominent for lung cancer than for other sites, and for lung cancer, more pronounced among males than among females. Such an effect could be produced solely by progressively improved recognition and diagnosis of lung cancer, and part of the shift may well be due to this factor. However, the magnitude of the change among males has been so great, even in the most recent years, that it would be hard to account for it completely in that manner, in view of the much smaller changes noted for female lung cancer and for other cancer sites.

To minimize the effect of improved diagnosis table 15 restricts comparisons to the most recent time period and contrasts the 1953 lung-cancer-mortality rate (used as an estimate for 1955) with the 1945 experience,



TEXT-FIGURE 1.—Death rates from cancer of the lung, bronchus, and pleura per 100,000 population between 1930 and 1950 for cohorts born in successive decades.

so that each cohort is compared at the same age with a cohort born in the preceding decade. Of the limited number of comparisons in table 15 the greatest differential for men appears in the contrast of the two oldest cohorts and narrows for the younger cohorts. Among women the ratios do not deviate significantly from 1 until the 65 to 74 year comparison between the cohorts born between 1880–89 and 1870–79 is reached.

TABLE 15.—*Lung-cancer-mortality rates per 100,000 population by age and sex. United States, 1945 and 1953*

Age	Males			Females		
	1953	1945	1953/1945	1953	1945	1953/1945
35–44 years	8.7	6.9	1.26	2.5	2.6	0.96
45–54 years	42.0	25.8	1.63	6.7	6.6	1.02
55–64 years	108.5	56.9	1.91	14.1	14.6	0.97
65–74 years	132.9	59.9	2.22	25.3	21.2	1.19
75–84 years	113.6	45.6	2.49	37.2	22.7	1.64

Discontinuance of regular smoking increases with advancing age and for this reason current cigarette-consumption rates as presented in table 2 *et seq.* are not a suitable index for drawing cohort comparisons. Maximum smoking rates attained are not influenced by the age effect of discontinuance, and table 16 presents the survey results on maximum rate of cigarette use for the sex-age groups. Dropping the relative risk of 3 for discontinued smokers, but otherwise applying the schedule of relative risks adopted for current rates to the corresponding category of maximum rates, the predicted cohort relationships of the rates are given in table 17. The inability to subdivide smoking-history distributions for persons born before 1890 into more detailed age groups limits the number of possible comparisons.

TABLE 16.—*Percentage distribution of population with respect to maximum cigarette-smoking rate attained, by sex and age**

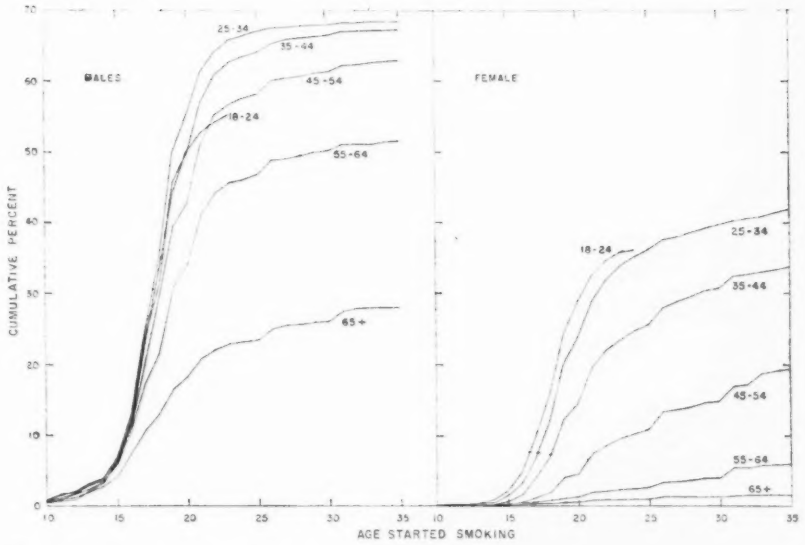
Sex and age	Maximum daily cigarette-consumption rate				
	Total	Never smoked cigarettes regularly	Less than 10 cigarettes	10 to 20 cigarettes	Over 20 cigarettes
Males					
35–44 years	100.0	26.7	8.5	38.4	26.4
45–54 years	100.0	30.9	8.9	36.7	23.5
55–64 years	100.0	41.1	10.0	31.0	17.9
65 years and over	100.0	65.1	9.2	17.7	8.0
Females					
35–44 years	100.0	63.0	11.7	21.0	4.3
45–54 years	100.0	73.9	8.6	13.4	4.1
55–64 years	100.0	86.9	5.1	6.2	1.8
65 years and over	100.0	95.2	2.4	2.1	0.3

**Note:* Data abstracted from Current Population Survey for February 1955 (*9*).

For each cohort comparison predicted in the first three lines of table 17 there is a corresponding observed ratio (for one age group only) in the first three lines of table 15. For men the predicted ratios fall below those observed, although there is agreement on the sequence of values for these ratios. Text-figure 2 illustrates the trend of younger males to take up cigarette smoking at earlier ages. Any modification of the model to state lung-cancer risk as a function both of age at which smoking was started and intensity would better the correspondence between the observed and predicted ratios for men. The effect of age at which smoking was started may become less important as duration of exposure is prolonged, and a more appropriate check on correspondence between prediction and observed fact may come in cohort comparisons at older ages than those shown in table 15. In view of the improved agreement between observation and prediction when the 55 to 64 age group was reached, the future course of the mortality rate for males 55 to 64 years would bear watching. If the model holds true, it would forecast lung-cancer mortality for the male population 55 to 64 years on the order of 128 and 138 per 100,000 population in 1965 and 1975, respectively, which would represent a slowing down of the past rate of increase. This projection can be recomputed when more current mortality rates are published and checked against the observed mortality trend for this age group in the next several years. If there are other carcinogenic agents to which younger cohorts are now

TABLE 17.—*Cohort ratios of lung-cancer rates predicted by model*

Year of birth	Males	Females
1910–19/1900–09	1.07 ± 0.02	1.24 ± 0.03
1900–09/1890–99	1.18 ± .03	1.49 ± .05
1890–99/1880–89	1.62 ± .06	1.44 ± .05
1910–19/1890–99	1.27 ± .03	1.85 ± .05



TEXT-FIGURE 2.—Cumulative percentage of persons becoming regular cigarette smokers prior to age 35, by sex and age.

being exposed more intensively, the observed increase would exceed the value predicted.

Text-figure 2 shows that only a few women were smoking cigarettes regularly prior to 1930. Any agent in cigarette smoke is at most a weak carcinogen, and a latent period of at least 15 years could reasonably be expected on the basis of exposures to known carcinogens, so that little effect in the cohort relationships for women attributable to smoking could have been expected prior to 1945. The rise which took place in the female lung-cancer rates prior to 1935 must be attributed to other factors, including increased recognition of the disease. Examination of the United States mortality data for more recent years shows that the lung-cancer rate for women 75 to 84 years did begin to rise by 1945 and has since continued to do so. The rise in the 75 to 84 rate has been accompanied by a more modest rise in the 65 to 74 year rate for women.

While the observed comparisons of mortality rates between the younger cohorts of women for the ages observed to date have not conformed to the predictions of the model, the question remains as to whether the predicted relationships will become operative at a later date. In view of the later ages at which women start to smoke and lower age-specific female mortality, which might result from a generally delayed response of women to the influence of an exogenous agent, the upturn in female lung-cancer mortality might be postponed to an older age group than has been noted for men.

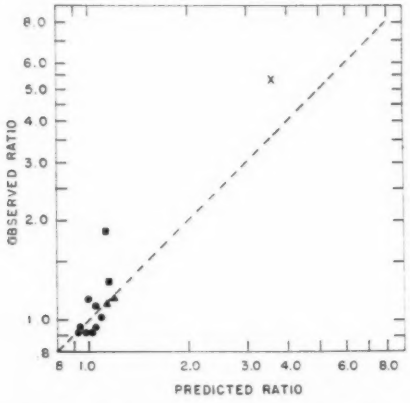
To cover this situation it would seem desirable to check the predicted cohort ratios against the mortality rates as later determined for the 65 to

74 age group. The model calls for female rates at ages 65 to 74 of about 36, 54, and 67 in 1965, 1975, and 1985, respectively (the projections can be adjusted when the final 1955 mortality data are available). Because of the pronounced trend to smoking at earlier ages among women born between 1910 and 1920 the model predicts that the 55 to 64 female rate will rise by 1965. An interesting feature, to be tested against future events, is that the model calls for an upturn in the female rates, simultaneous with a slowing down of the rates of increase for male lung-cancer mortality.

Discussion

Some analyses for compatability between data on smoking patterns and on the epidemiology of lung cancer in the population of the United States, using a schedule of relative risk to lung cancer derived from special retrospective and prospective studies on the subject, have been presented. As pointed out in the introduction, this material is not a *de novo* line of evidence, but a determination of whether data from the special studies may be applicable to the general population.

Text-figure 3 summarizes the comparisons based on broad population groupings drawn from tables 1, 3, 6, 8, 9, 11, 12, and 14. To give equal weight to percentage deviations of ratios from the expected values, a log scale is used. The individual points plotted indicate the agreement between the observed ratios of lung-cancer rates and the ratios predicted by the model of relative risk under investigation and the extent of departure from the linear relationship of exact correspondence between observation and prediction. It can be seen that these points are reasonably close to the linear regression. If the disparity in smoking patterns between two population groups being compared is small, the observed lung-cancer differences tend to be small, and *vice versa*. The two major deviations from a straight line occur in the sex ratio and in the male urban-



TEXT-FIGURE 3.—Summary of observed and predicted ratios of lung-cancer rates derived from comparing population groups with respect to sex (×), urban-rural residence (■), color (▲), and region (●).

rural ratio. The deviations have been discussed in the text. It is our belief that the sex-ratio deviation may well represent a basic male-female difference in mortality from neoplastic disease in general and from many non-neoplastic causes as well. The urban-rural discrepancy, in our opinion, represents a real finding and is a manifestation of multiple environmental factors in lung cancer. Both deviations fit well into a general hypothesis of lung-cancer epidemiology, and a closer fit to a straight-line relationship might be more suspect than the deviations.

The weight of the evidence as summarized in text-figure 3 favors the conclusion that the excess lung-cancer risk among cigarette smokers, demonstrated in special study groups, occurs in the total population as well. At least the available evidence is compatible with this interpretation, and no alternative interpretations seem reasonable for the observed facts. The possibility that the results in text-figure 3 based on group comparisions could be produced by self-selection seems remote. Under this hypothesis each population subgrouping would have its own quota of persons predisposed toward smoking. Any effects attributable to smoker self-selection of individuals, which may be associated with unspecified biologic characteristics, should operate within each group and thus tend to cancel out in group comparisons, unless large intergroup differences in the proportions subject to self-selection exist. Sex and race differentials in the supposed biologic characteristics governing self-selection are conceivable, but it is hard to see why this should extend to comparisons of urban and rural residents, in view of the considerable population interchange and mixture through migration.

Additional studies that would further test the model on the relationship of smoking to lung cancer have been mentioned in the text. These suggestions were chiefly concerned with extensions of observations now available. Tests based on further refinements of the model of relative risk to take account of histologic type might be added. Data on the relative risks for and distribution of each histologic type are fragmentary, but seem to be consistent with the other features presented here.

The approach in this paper is but one of several ways in which the interpretation of the reported association between smoking and lung cancer may be tested. Some alternative methods have been discussed (*27*). In view of the practical importance of working out the epidemiologic details of lung cancer, some of the alternate lines of investigation should be pressed. Two seem to be of most immediate importance. The first is the use of retrospective and forward studies to establish by direct observation among women the nature of the relative risks by smoking class for that sex. The present material merely asserts that the known facts on lung-cancer rates for women are not inconsistent with the pattern established by observation on men. The second is to observe groups in which smoking does not form part of the cultural pattern, to see whether their lung-cancer-mortality experience is less than that for an appropriate segment of the United States population in a degree commensurate with differences in smoking patterns. The latter would be a substitute for

a direct test through application of control measures, one of the accepted methods for identifying an association as being etiologically significant.

Summary

This investigation was designed to test whether the results of special retrospective and prospective studies were compatible with more broadly based information on the distribution of lung cancer. A schedule of relative lung-cancer risks by smoking class was adapted from these special studies and applied to distributions of cigarette-smoking patterns in the United States population as found in a recent survey. The observed sex, urban-rural, white-nonwhite, and regional ratios of lung-cancer mortality and morbidity were compared with those predicted by the model adopted for investigation. Correspondence between the observed facts and the predictions was good and the results do not contraindicate generalization from the special-study findings on excess risk of lung cancer among cigarette smokers to the total population.

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Enzyme Changes Induced in Normal and Malignant Tissues with Chemical Agents. VI. Effect of Acetylpodophyllotoxin-ω-Pyridinium Chloride on Malic-Oxidase and on Isocitric-Oxidase Systems of Sarcoma 37[1,2]

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In a previous communication from this laboratory (*2*) it was shown that a single subcutaneous injection of acetylpodophyllotoxin-ω-pyridinium chloride (NCI-3022) produced a marked decrease in succinoxidase activity of sarcoma 37, with little or no effect on the succinic dehydrogenase activity. It was pointed out that the phenomenon was similar to the one suggested by Slater (*3*) in his work on BAL (British antilewisite), and a factor was postulated that appears to be the site of action for a number of agents (*3–6*) that inhibit oxygen uptake in a complete system. Slater (*7*) and Reif and Potter (*6*) have indicated that this factor is also a component of the system between reduced diphosphopyridine nucleotide (DPNH) and cytochrome c.

Whether such a phenomenon occurs in the reaction systems involving diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) and cytochrome c in the oxidation of tumor homogenates from drug-treated animals was next investigated.

In the present report, the oxidation of malic acid in the presence of DPN and cytochrome c and the oxidation of *d*-isocitrate in the presence of TPN and cytochrome c have been studied, and the effect of tumor-damaging agents on the enzymes that participate in the oxidation of malic acid and *d*-isocitric acid was investigated.

Methods and Materials

Acetylpodophyllotoxin-ω-pyridinium chloride (NCI-3022), a soluble derivative of podophyllotoxin (*8*), was employed in these experiments. CAF_1 [(BALB/c × A)F_1] hybrid mice bearing 6-day intramuscular implants of sarcoma 37 received a single subcutaneous injection of 20 μg. of the compound (NCI-3022) per gm. and were sacrificed at intervals from 2 to 24 hours after injection. The tissues of at least four animals were pooled for each time interval for analysis. Each of the points on the

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[2] Presented in part at the annual meeting of the American Association for Cancer Research, Inc., April 14–17, 1955, San Francisco, Calif. (*1*).
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[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

figures and each of the values given in the tables represents the average of two separate experiments. Homogenates of normal and tumor tissues were prepared for enzyme studies as described (*9*).

DPN, DPNH (approximately 90%), TPN and TPNH (90–95%), and cytochrome c used in these experiments were obtained from Sigma Chemical Company.

Malic-oxidase activity of tissue homogenates was determined according to the method of Potter (*10*) and malic dehydrogenase as described by Sacktor (*11*) with the use of 0.5 ml. DPN, 0.7 μM; 0.1 ml. KCN 0.4 μM; 0.5 ml. malic acid, 0.09 M; 1.4 ml. tris buffer, 0.1 M, *p*H 9.0; enzyme and water to make 3 ml. The blank contained all the components except malic acid. Changes in the optical density at 340 mμ. at 20-second intervals were observed with a Beckman DU spectrophotometer at 23°, and the amount of DPNH formed was calculated by employing an extinction coefficient of 6.27×10^6 sq. cm. per mole for DPNH (*12*).

Dihydrocozymase (DPNH) oxidase system was followed according to Slater (*7*) by determining at 30-second intervals the decrease in optical density at 340 mμ. of 0.5 ml. DPNH, 0.43 μM, in phosphate buffer 0.1 M, *p*H 7.4; 0.2 ml. cytochrome c, 0.4 μM, and an appropriate dilution of the homogenate. Malic-cytochrome c reductase activity was measured as detailed by Sacktor (*11*). The reaction mixture contained 0.3 ml. cytochrome c, 0.4 μM; 0.1 ml. KCN, 0.002 M; 0.5 ml. malic acid, 0.09 M, *p*H 7.4; 1.0 ml. phosphate buffer, 0.1 M, *p*H 7.4; 0.1 ml. DPN, 0.7 μM; and tissue homogenate. The blank contained all components except the tissue homogenate. The rate of reduction of cytochrome c was read every 30 seconds at 550 mμ. The determination of DPN-cytochrome c reductase was carried out according to the procedure of Hogeboom and Schneider (*13*).

In some experiments such as malic dehydrogenase and DPNH oxidase the homogenates were passed through a tissue press at 20,000 psi (pounds per sq. inch) pressure at 3° (*14*) to obtain the maximum enzyme activity. Activity of homogenates passed through a press increased in enzyme activity by 2 to 3 times over that of the original homogenate.

Isocitric dehydrogenase, TPN-cytochrome c reductase, and oxidation of *d*-isocitrate by the homogenates of liver and tumor from the drug-treated animals were carried out according to the procedure of Hogeboom and Schneider (*15*). Malic-dehydrogenase and isocitric-dehydrogenase activity of the control and treated-tumor homogenates were also carried out in presence of methylene blue and potassium cyanide. The ingredients for both reactions were the same as those used for malic oxidase or for the oxidation of isocitrate reaction except that cytochrome c was replaced by 0.4 ml. of 0.01 M methylene blue and 0.3 ml. of 0.01 M KCN (neutralized).

Results

Table 1 gives the results of the effect of a single subcutaneous injection of 20 μg. of NCI-3022 per gm. on malic-oxidase and malic-dehydrogenase

activity of homogenates of sarcoma 37 and of liver. Over 30 percent of the malic-oxidase activity was lost within 4 hours after injection and at 24 hours almost all activity disappeared. On the other hand, malic-dehydrogenase activity of the tumor was not affected by the action of the drug. The changes in both enzyme activities of the liver from drug-treated animals were very small.

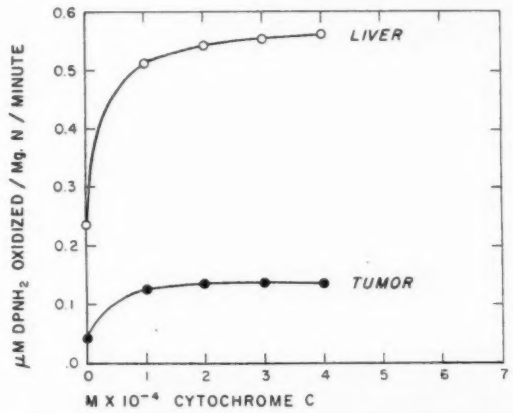
TABLE 1.—*Effect of a single subcutaneous injection of 20 μg. of NCI-3022 per gm. on malic-oxidase and malic-dehydrogenase activity of sarcoma 37 and of liver*

Time after injection (*hours*)	Residual activity *†			
	Malic oxidase		Malic dehydrogenase	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	89	106	107	97
4	69	102	103	108
6	66	99	114	102
24	7	98	112	111

* $\frac{\text{Activity of treated}}{\text{Activity of control}} \times 100$.

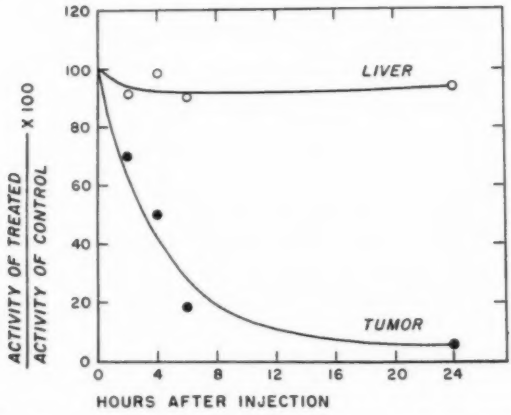
† Control values.—For malic oxidase Q_{O_2} (mm.³ O_2 per mg. N per hr.). Tumor: 166–171; liver: 561–571. For malic dehydrogenase (micromoles of DPN reduced per mg. N per min.). Tumor: 0.225–0.227; liver: 0.926–0.994.

Slater (7) has suggested that cytochrome c is a component of DPNH oxidase, and the maximum concentration of cytochrome c to be added to the system was determined accordingly. Text-figure 1 shows the effect of added cytochrome c to the medium for DPNH-oxidase system of sarcoma 37 and of liver. With the addition of $2.67 \times 10^{-5}M$ (0.2 ml. of



TEXT-FIGURE 1.—Effect of addition of cytochrome c (0.2 ml.) to reaction mixture (3 ml.) for DPNH-oxidase activity of sarcoma 37 and of liver.

4×10^{-4} *M*) cytochrome c the activity increased with both tissues by 2 to 3 times over that when cytochrome c was not added to the medium. The effect of 20 μg. of NCI-3022 per gm. on DPNH-oxidase of sarcoma 37 and of liver is shown in text-figure 2. There was a progressive loss in the enzyme activity of the tumor at all intervals and practically all the activity was lost at 24 hours after injection. During this time the enzyme activity of the liver was scarcely affected.

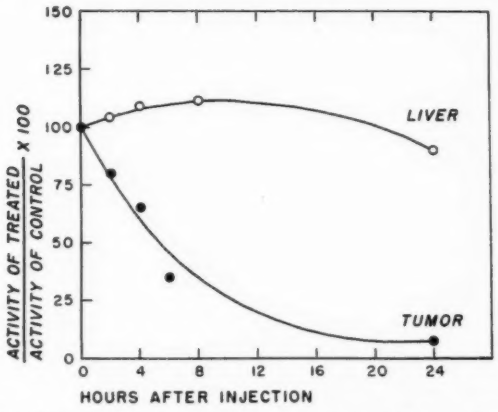


TEXT-FIGURE 2.—Effect of NCI-3022 (20 μg./gm.) on DPNH-oxidase activity of sarcoma 37 and of liver. Range of control values—micromoles of DPNH oxidized per mg. N per minute.

Tumor: 0.131–0.137
Liver: 0.523–0.553

The ability of the tumor and liver from mice treated with 20 μg. of NCI-3022 per gm. to reduce cytochrome c in the presence of malic acid is shown in text-figure 3. The pattern of decrease of the enzyme activity of tumor homogenate was similar to that of malic-oxidase activity of the tumors from treated animals. Insignificant changes in the enzyme activity of liver were observed. The malic-cytochrome c reductase activity of the tissues increased with the addition of DPN to the reaction mixture up to approximately 6×10^{-4} *M* concentration (text-fig. 4). This finding was similar to that reported by Sacktor (*11*) on the enzyme activity of insect (housefly) mitochondria.

The changes in DPN-cytochrome c reductase activity of tumor and of liver from animals treated with NCI-3022 are given in text-figure 5. The compound produced a marked drop in the enzyme activity of the tumor, whereas the enzyme activity of the liver remained practically constant throughout the experiment. Results on the effect of the compound NCI-3022 on isocitric-oxidase and isocitric-dehydrogenase activity of the tumor and liver are given in table 2. Marked changes were observed in the isocitric-oxidase activity of the tumor, whereas the isocitric-dehydrogenase activity showed little change. These results were similar

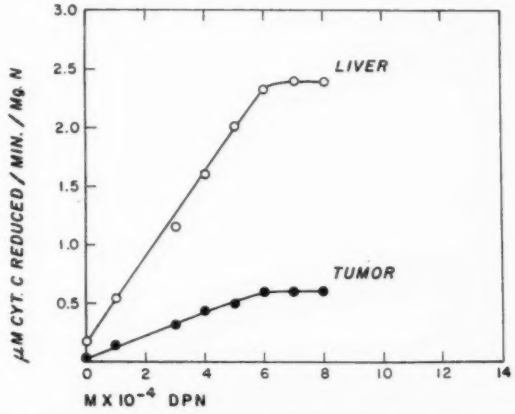


TEXT-FIGURE 3.—Effect of NCI-3022 (20 μg./gm.) on malic-cytochrome c reductase activity of sarcoma 37 and of liver. Range of control values—micromoles of cytochrome c reduced per mg. N per minute.

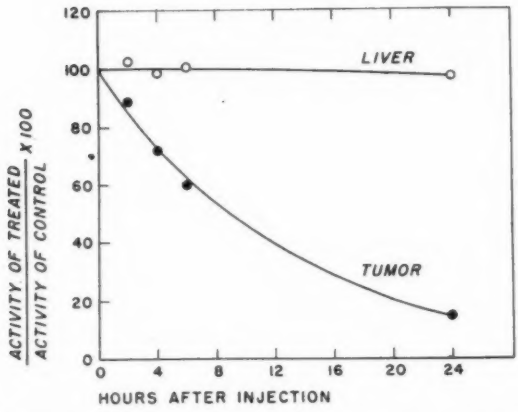
Tumor: 0.59–0.60
Liver: 2.37–2.38

to those obtained with malic-oxidase and malic-dehydrogenase activity of the drug-treated tumors. As before, the changes in the oxidase and dehydrogenase activity of the liver from the chemically treated animals were not significant.

Since both TPN and cytochrome c are involved in the oxidation of isocitrate, the rate of reduction of cytochrome c in the presence of dihydrotriphosphopyridine nucleotide (TPNH) by the tissue was next investigated. It was found (text-fig. 6) that TPN-cytochrome c reductase activity of tumor from animals treated with NCI-3022 dropped below 50 percent of the controls in 4 hours, and by 24 hours all the activity



TEXT-FIGURE 4.—Effect of addition of DPN (0.1 ml.) to the reaction mixture (3 ml.) on malic-cytochrome c reductase activity of sarcoma 37 and of liver.



TEXT-FIGURE 5.—Effect of NCI-3022 (20 μg./gm.) on DPN-cytochrome c reductase activity of sarcoma 37 and of liver. Range of control values—micromoles of cytochrome c reduced per mg. N per minute.

Tumor: 0.49–0.52
Liver: 1.97–2.07

TABLE 2.—*Effect of a single subcutaneous injection of 20 μg. of NCI-3022 per gm. on isocitric-oxidase and isocitric-dehydrogenase activity of sarcoma 37 and of liver*

Time after injection (*hours*)	Residual activity*†			
	Isocitric oxidase		Isocitric dehydrogenase	
	Tumor (*percent*)	Liver (*percent*)	Tumor (*percent*)	Liver (*percent*)
2	93	99	95	98
4	74	98	106	92
6	52	96	109	102
24	10	90	102	99

*$\frac{\text{Activity of treated}}{\text{Activity of control}} \times 100$.

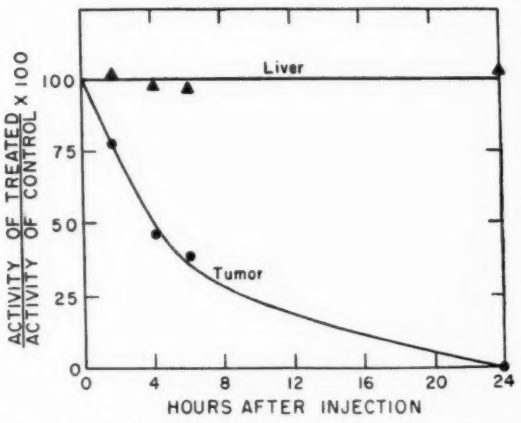
†Range of control values.—For isocitric oxidase Q_{O_2} (mm.³ O_2 per hr. per mg. N). Tumor: 40.5–43.5; liver: 330.9–338.5. For isocitric dehydrogenase (micromoles of TPN reduced per min. per mg. N). Tumor: 0.0491–0.0492; liver: 0.790–0.829.

disappeared in these tumors. The enzyme activity of the liver from the injected animals showed only minor changes.

Discussion

The data presented here show that malic-oxidase activity of sarcoma 37 from the drug-treated animals decreased markedly within 24 hours after injection, whereas malic-dehydrogenase activity was hardly affected at these intervals after the injection of the compound.

In the treated tumors other systems of enzymes that are closely associated with the malic-oxidase system, such as DPNH-oxidase, malic-



TEXT-FIGURE 6.—Effect of NCI-3022 (20μg./gm.) on TPN-cytochrome c reductase activity of sarcoma 37 and of liver. Range of control values—micromoles of cytochrome c reduced per mg. N per minute.
Tumor: 0.060–0.064
Liver: 0.263–0.274

cytochrome c reductase, and DPN-cytochrome c reductase activity, showed rate reductions similar to that of malic-oxidase activity.

The results on malic oxidase and malic dehydrogenase are comparable to our previous findings on succinoxidase and succinic-dehydrogenase systems, when the succinoxidase activity of the treated tumor was markedly affected while the succinic-dehydrogenase level was only slightly affected. Similarly, the oxidation of *d*-isocitrate by tumor homogenate from drug-treated animals was markedly lowered; however, under the same conditions the activity of isocitric dehydrogenase was scarcely affected. The TPN-cytochrome c reductase activity of the tumor was lowered considerably by the action of the drug.

These observations suggest that the component postulated by Slater (*3,7*) to play a part in the succinoxidase system and DPNH-oxidation reaction also might take part as a component of the DPN-linked malic-oxidase system and of the TPN-linked isocitric-oxidase system. However, preliminary experiments on the *in vitro* effect of BAL on the malic-oxidase and on the succinoxidase activity of sarcoma 37 have shown that only about 50 percent of the original malic-oxidase activity but almost 100 percent of the succinoxidase activity of the tumor homogenate was inhibited by incubating with 0.01 *M* and 0.03 *M* BAL for half an hour at 37.5° C. Similarly, these concentrations of BAL inhibited the isocitric-oxidase activity of the tumor to an extent of only 25 to 50 percent of the original activity. These experiments suggest the existence of an alternate pathway for DPN and TPN oxidation that by-passes the factor that allows complete inhibition of the succinoxidase system by BAL. This observation is similar to that reported by Potter and Reif with antimycin A (*16*).

Although a marked effect of the drug was observed on the oxidase systems, especially on the succinoxidase system of the tumors as was reported previously (*2*), this effect does not appear to be specific in nature as in the case with BAL (*3*). The criteria postulated by Slater (*17*) are of importance in determining whether the drug has a general nonspecific effect on the succinoxidase system or is acting specifically on a single component of the system. For nonspecific action, the inhibition of the complete succinoxidase system is greater in the presence of added cytochrome c than that in the absence of added cytochrome c. Secondly, for a nonspecific effect the inhibition can be reversed by the addition of agents such as $Ca_3(PO_4)_2$ gel to the enzyme system, which remove the inhibitor by absorption or in some unknown way.

In some of our preliminary studies on succinoxidase activity of the tumors from NCI-3022-treated animals we have observed that the loss in enzyme activity when evaluated in the presence of added cytochrome c was much greater than that observed in enzyme determination without addition of cytochrome c. When $Ca_3(PO_4)_2$ gel was used in these enzyme determinations with added cytochrome c to restore the loss in enzyme activity due to drug, it was found that about 30 to 40 percent of the lost activity was recovered.

Decreases in succinoxidase activity of the tumors from the drug-treated animals in the absence of added cytochrome c were scarcely noticeable, even up to 6 hours after injection of the drug. It was only at 24 hours after injection, when the tumors were necrotic, that the bulk of the activity was lost.

The enzyme activity measured in the absence of added cytochrome c but in the presence of $Ca_3(PO_4)_2$ gel showed practically no loss in the activity of these tumors. Here again the bulk of the activity was lost at 24 hours after injection.

Although the partially purified preparations used by Slater were probably particulates of mitochondrial origin, whereas the experiments referred to in this paper were performed on homogenates, it would appear that the effect produced by the drug on succinoxidase activity of the tumors is of a nonspecific nature and is intimately involved in the cytochrome system rather than with the specific dehydrogenases. Further work, along with the studies on other oxidase systems, is in progress.

Summary

1) Homogenates of sarcoma 37 from mice that had received a single subcutaneous injection of 20 μg. of acetylpodophyllotoxin-ω-pyridinium chloride per gm. showed a marked decrease in malic-oxidase activity. At 24 hours after injection most of the activity was lost, whereas malic-dehydrogenase activity was scarcely affected.

2) In the tumor, other enzyme systems that are closely associated with the malic-oxidase system, such as malic-cytochrome c reductase, DPN-cytochrome c reductase, and DPNH oxidase, were equally affected by the drug.

3) Addition of cytochrome c enhanced the DPNH-oxidase activity of both treated and untreated tissues in proportion to their respective activities. Similarly, addition of DPN increased the malic-cytochrome c reductase activity of the tissues.

4) Oxidation of *d*-isocitrate by the homogenates of tumors from drug-treated animals was markedly lowered while isocitric dehydrogenase was not affected. Other components of this system such as TPN-cytochrome c reductase activity of the treated tumor also showed marked decrease in activity.

5) Preliminary evidence was presented to show that the effects of the tumor-damaging agent on the specific oxidase systems (succinic, malic, and isocitric) could be accounted for by the action on the cytochrome system.

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Interrelations in Respiratory, Phosphorylative, and Mitotic Activity of Ehrlich Ascites-Tumor Cells: Influence of Stilbestrol and Quinone[1]

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The division of mammalian cells appears to be dependent on aerobic conditions and a source of energy, presumably derived from phosphate bonds (*1,2*). Evidence for the direct utilization of high-energy phosphate, synthesized during the Krebs cycle, for division of marine eggs was recently presented by Barnett and Downey (*3*). The significance of respiration in mitosis, whether oxygen exerts an influence other than its utilization indirectly for the oxidative generation of energy sources, remains obscure.

In an attempt to gain further insight into the interrelationships between the respiratory, phosphorylative, and mitotic activity of cells, Ehrlich ascites-tumor cells were exposed to agents influencing these activities, and the relative changes produced were compared. A previous report, describing the influences of 2,4-dinitrophenol (DNP), indicated that the rate of cell division may be independently related to both the respiratory rate and the availability of high-energy phosphate (*4*). A similar observation was made in the present experiments, in which the effects of diethylstilbestrol (stilbestrol) and benzoquinone (quinone) were determined.

Stilbestrol, widely used in recent years as a synthetic estrogen, has been employed in the chemotherapy of human prostatic and breast cancers (*5*). At the cellular level, stilbestrol inhibits dehydrogenase systems, perhaps acting as a competitive intracellular hydrogen carrier in the form of stilbestrol quinone (*6*). In its action on yeast metabolism stilbestrol behaves similarly to DNP, markedly stimulating endogenous respiration at low concentrations and inhibiting at higher reagent levels (*7*). In view of its influences on cellular functions, it was considered of interest to determine the action of stilbestrol on Ehrlich ascites-tumor cells, as previously carried out with DNP. Since stilbestrol may act in the form of its quinone, the influence of a simple compound of this type was also determined.

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Materials and Methods

The methods and conditions used for the processing of tumor cells, for measurement of oxygen utilization, and for separation and assay of cellular phosphate fractions were identical to those described previously (*4*). Ehrlich ascites-tumor cells, harvested 7 days after inoculation into C3H mice, were washed four times in Krebs-Ringer's phosphate solution. Respiration of 0.2 ml. aliquots of packed cells, suspended in Krebs-Ringer's phosphate and tris buffer, was determined at 37° C. and *p*H 7.4 by standard Warburg techniques. When employed, stilbestrol (dissolved in a minimal amount of alkali), quinone, and P^{32} (approximately 25 μC. in neutral isotonic solution) were added to the cell suspension at zero time, following a 10-minute equilibration period.

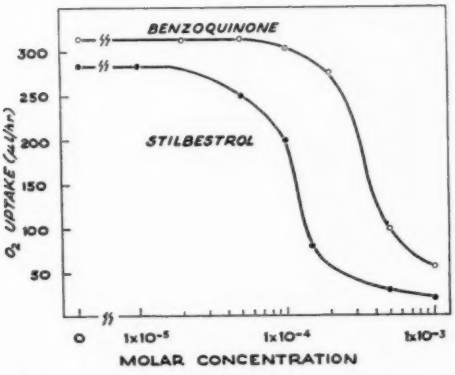
At the end of the 1-hour respiratory period, the contents of duplicate vessels were combined and the cells were separated from the "medium" by centrifugation. Since there appeared to be no appreciable contamination of cells with P^{32} from the medium (*4*), the cells were subjected to the phosphate-fractionation procedure without further washing. Acid-soluble extracts of both cells and medium were prepared with cold trichloroacetic acid. Inorganic-phosphorus (IP) and labile-phosphorus (LP) fractions were separated from the extracts; the remainder, containing non-nucleotide acid-soluble organic phosphate, was characterized as ester phosphorus (EP). Deoxyribonucleic-acid phosphorus (DNAP) was isolated from the acid-insoluble cellular residue by a modified Schmidt-Thannhauser partition.

All phosphorus contents are shown as μg. per ml. of cells, or fluid associated with 1 ml. of cells. Radioactivity of IP fractions was measured in terms of specific activity (S.A.), defined as the ratio of percentage counts added to mg. phosphorus per ml. Activity of other fractions is expressed as relative specific activity (R.S.A.), the percentage ratio of S.A. of the fraction to that of the corresponding IP fraction.

Results

The effects of graded concentrations of stilbestrol and quinone on the respiration of washed Ehrlich ascites-tumor cells are shown in text-figure 1. Increasing concentrations of the reagents had only an ever-increasing inhibitory effect on ascites-cell respiration, as opposed to their influence on the endogenous respiration of yeast, in which a stimulatory phase was also apparent (*4*). Since moderate amounts of added glucose inhibit the respiration of washed Ehrlich ascites-tumor cells (*8,9*), the endogenous glucose content of the cells would appear to be near optimal levels. It may not be surprising, therefore, that the effects of stilbestrol and quinone, as well as DNP (*4*), on respiration of Ehrlich ascites-tumor cells resembled more closely those observed on exogenous glucose respiration of yeast, rather than on endogenous respiration.

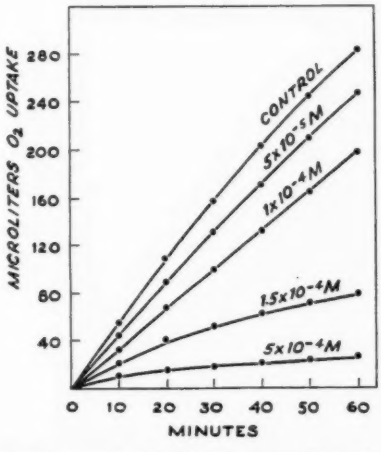
As shown in text-figure 2, stilbestrol exerted an immediate effect on the respiration of Ehrlich ascites-tumor cells at all concentrations, behaving like DNP in this respect (*4*).



TEXT-FIGURE 1.—Effect of graded concentrations of stilbestrol and benzoquinone on O_2 uptake of Ehrlich ascites-tumor cells. Each flask contained 0.2 ml. of packed cells. Reagent concentrations are final levels after tipping in of sidearm contents.

The influence of graded concentrations of stilbestrol on phosphorus relationships of the cells is presented in table 1. With increasing concentrations of stilbestrol, both the content and activity of cellular IP remained relatively unchanged until a precipitous fall in respiration occurred. IP content then increased, while the specific activity fell markedly. With further increase in stilbestrol concentration to $5 \times 10^{-4} M$ the trend was reversed, and cellular-IP content fell below control levels. The action of stilbestrol on cellular IP contrasted with that of DNP, which evoked an immediate and sustained increase in cellular IP with increasing concentration (*4*).

Cellular LP and EP contents also remained relatively uninfluenced by stilbestrol at lower reagent concentrations. As inhibition of respira-



TEXT-FIGURE 2.—Time-rate curves of ascites-tumor-cell respiration as influenced by stilbestrol.

TABLE 1.—*Influence of stilbestrol on phosphorus relationships of ascites-tumor cells**

Stilbestrol concentration	IP		LP		EP		DNAP	
M	μg./ml.	S.A.†	μg./ml.	R.S.A.‡	μg./ml.	R.S.A.‡	μg./ml.	R.S.A.‡
				Cells				
0	309	68. 9	134	96. 0	446	34. 2	524	0. 169
1×10^{-5}	292	69. 3	122	103. 5	488	25. 9	511	0. 154
5×10^{-5}	290	67. 1	124	91. 3	407	30. 1	541	0. 139
1×10^{-4}	295	61. 6	117	71. 6	396	20. 5	504	0. 086
$1. 5\times10^{-4}$	387	29. 9	41	23. 5	356	16. 4	540	0. 005
5×10^{-4}	207	35. 7	23	25. 9	230	17. 5	541	0. 003
				Medium				
0	326	128	10	—	86	—		
1×10^{-5}	334	127	17	—	71	—		
5×10^{-5}	354	128	7	—	79	—		
1×10^{-4}	418	120	11	—	66	—		
$1. 5\times10^{-4}$	515	126	6	—	83	—		
5×10^{-4}	758	86	6	—	241	—		

*Typical values from one of four similar experiments.
†Specific activity = percent counts added per mg. P per ml.

‡Relative specific activity = $\frac{\text{S.A. fraction}}{\text{S.A. IP}} \times 100$.

tion became intensified, however, both LP and EP diminished markedly. Suppression of P^{32} uptake into LP with increasing stilbestrol concentration paralleled inhibition of respiration. DNP, in contrast, accelerated breakdown of the organic phosphates at low concentration, while stimulating respiration (*4*).

Cellular DNAP content remained constant throughout the range of stilbestrol concentrations used. Uptake of P^{32} into DNAP, however, was increasingly suppressed as the stilbestrol concentration was increased, indeed to a greater extent than respiration. At no level of stilbestrol was there any augmentation of P^{32} uptake into DNAP, as was observed with DNP.

Medium IP increased progressively as the stilbestrol concentration was increased. At a concentration of 5×10^{-4} *M*, there was a marked outpouring of IP into the medium, and it was only at this stilbestrol level that medium EP rose. Radioactivity of medium IP remained constant until the highest level of stilbestrol was attained, falling abruptly coincident with the sudden rise in IP content.

The action of benzoquinone on phosphate relationships of Ehrlich ascites-tumor cells in all respects resembled that of stilbestrol.

Discussion

Stilbestrol is a known inhibitor of respiratory enzyme systems (*10*); it seems likely, therefore, that the respiratory inhibition of Ehrlich ascites-tumor cells may also be due to such action. Alternatively, however, it is possible that some of the inhibitory action of stilbestrol on cellular processes may be due to membrane phenomena, as had been suggested earlier in connection with DNP action on the ascites-tumor cells. That

such phenomena occur became evident as the reagent concentration was increased from 1.5×10^{-4} *M* to 5×10^{-4} *M*. The concentration of cellular IP, which had become elevated at 1.5×10^{-4} *M*, probably as a result of the breakdown of LP and EP, decreased to its lowest level in spite of continued breakdown of organic phosphorus. The decrease in cellular IP was reflected by a surge in the release of IP into the medium and a fall in the specific activity of medium IP. Concentration of medium EP, which had remained relatively constant, rose abruptly. Moreover the cellular content of lactic acid, after having risen steadily as the stilbestrol level was raised to 1.5×10^{-4} *M*, fell at 5×10^{-4} *M*.[4] These effects can probably be taken as overt evidence of increased membrane permeability, and may perhaps be suggestive of more subtle membrane phenomena occurring at lower reagent concentrations. It is of interest in this connection that Tateno and Kilbourne (*11*) have observed stilbestrol-induced hemolysis of human erythrocytes *in vitro*.

Cellular mitotic activity, as indicated by the uptake of P^{32} into DNAP, appeared to be independently related to both the rate of O_2 uptake and the availability of high-energy phosphorus with stilbestrol as the influencing agent as well as with DNP (*4*). Thus, the products of O_2 uptake and the ratio of LP : DNAP P^{32} (both activities measured in total counts per minute per ml. of cells) at each reagent level were roughly comparable until the highest concentration was reached. At 5×10^{-4} *M* stilbestrol, perhaps associated with the marked effect on cellular permeability, the relationship no longer seemed to hold.

It is interesting to compare the *in vitro* effects of stilbestrol with *in vivo* effects of the agent. Sugiura (*12*) observed no effect of subcutaneous injections of diethylstilbestrol dipropionate on the growth of Ehrlich ascites tumor, while Creech *et al.* (*13*) obtained similarly negative results on survival of tumor-bearing mice receiving stilbestrol intraperitoneally. Davidson and Freeman (*14*) noted inhibition of *in vivo* incorporation of P^{32} into tumor DNA of mice bearing adenocarcinoma 755 after the mice had been treated with stilbestrol for several days. LePage and Greenlees (*15*) found partial inhibition of glycine-2-C^{14} incorporation *in vitro* into Ehrlich ascites-carcinoma cells exposed to 1.6×10^{-4} *M* stilbestrol. Heidelberger and Keller (*16*) noted no effect on the *in vitro* incorporation of P^{32} into tumor DNA of rats bearing Flexner-Jobling carcinoma after administration of stilbestrol. Approximately 40 percent inhibition of incorporation of radioactive formate into DNA adenine and guanine occurred however. In general, it would appear that stilbestrol had little effect on tumor growth or survival of tumor-bearing animals. There may, however, be some inhibition of incorporation of precursors into tumor nucleic acids, both *in vitro* and *in vivo*.

Summary

The effects of graded concentrations of diethylstilbestrol and benzoquinone on interrelationships between respiration and phosphorylations

[4] Unpublished data.

of Ehrlich ascites-tumor cells were studied *in vitro*. With increasing concentration, both reagents progressively inhibited cellular respiration. Changes in acid-soluble phosphate fractions occurred only at high reagent levels. Incorporation of P^{32} into deoxyribonucleic-acid phosphorus appeared related to both the respiration rate and the availability of high-energy phosphorus. At high concentrations stilbestrol and quinone markedly affected the permeability of the cellular membrane.

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Certain Effects of *p*-Dimethylaminoazobenzene, Deficient Diet, and Hypotonic Media on Mitochondrial Enzymes in Experimental Rat-Liver Carcinogenesis [1]

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The modifications of mitochondrial enzymic activities during experimental liver carcinogenesis by aminoazo-dye feeding may depend either upon direct action of the carcinogenic agent or deficient diets or upon their interaction. However, recent investigations (*1,2*) have emphasized a close correlation between the morphology of the isolated mitochondria and their enzymic activities. It is therefore possible that some enzymes may be influenced, during carcinogenesis, through modifications of the mitochondrial structure. Some of these modifications can be demonstrated by the use of hypotonic media, which usually cause a remarkable swelling of the normal mitochondrial bodies (*3*).

Consequently it seemed desirable to study the influence of some deficient diets, of *p*-dimethylaminoazobenzene (*p*-DAB), and of isotonic and hypotonic media, and their interactions, on some mitochondrial enzymes during experimental liver carcinogenesis.

Two mitochondria-bound enzymes, cytochrome c oxidase and adenosinetriphosphatase (ATPase), and one diffusible enzyme, acid phosphatase, were tested with isotonic and hypotonic media differing by about 4.8 atmospheres, *i.e.*, the widest possible range in a vertebrate (*4*); all effects from the osmotic reactivity of the mitochondria during liver carcinogenesis should fall within this range, since a greater difference is hardly compatible with life. Morphologic analysis of the isolated liver mitochrondria has been undertaken by means of the electron microscope.

Materials and Methods

Experimental Animals

Two hundred adult albino rats of either sex, weighing about 200 gm., were fed the following diets for 4 months:

1. *Control diet* (Coward diet): corn, 65%; flour, 20%; casein, 9%; dry baker's yeast, 5%; NaCl, 0.5%; $CaCO_3$, 0.5%; plus fresh vegetables. This group is designated as "Controls."

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2. *Coward diet*+p-*DAB*: control diet plus *p*-DAB in a final concentration of 0.06%. This group is designated as "Coward diet+*p*-DAB."
3. *Deficient diet:* polished rice plus cod-liver oil, 20 mg. per kg. This group is designated as "Deficient diet."
4. *Cantero diet:* deficient diet with addition of *p*-DAB (3% in cod-liver oil) to a final concentration of 0.06%. This group is designated as "Cantero diet."

Each rat in groups 3 and 4 was given 1 gm. of fresh carrot per day, and 300 μg. of vitamin B were injected subcutaneously every month to prevent a polyneuritic syndrome.

Twelve hours prior to the experiment food was withdrawn. The rats were stunned by a blow on the back of the head and then decapitated. The liver was removed as quickly as possible for histologic examination and biochemical investigations.

Liver Fractionation

Once removed, the liver was immediately homogenized in 0.25 *M* cold sucrose + 0.005 *M* Versene (1 part of liver to 4 parts of solution). The fractionation was carried out following the main lines of the Schneider technique (*5*), with a refrigerated centrifuge model Phywe (made by A. G. Phywe, Göttingen, Germany), and the temperature was maintained between 0 and −4° C. during the whole procedure. The nuclei were centrifuged at 900 × g for 10 minutes and the supernatant was discarded. The sediment, resuspended in isotonic solution, was again centrifuged at 8,500 × g for 10 minutes. The supernatant was again discarded, and the mitochondria were suspended either in hypotonic or isotonic sucrose for 40 minutes. They were then centrifuged at 10,000 × g for 10 minutes. The sediments, resuspended in isotonic or hypotonic solutions, were labeled "isotonic mitochondria" and "hypotonic mitochondria," respectively. The supernatants were labeled in the same way. The washings of the nuclear fraction were omitted to shorten the procedure, in order to avoid an excessive decline of the enzymic activity with time. The homogeneity of the mitochondrial fraction was always checked by phase-contrast microscopy.

Osmotic Pressure

The isotonic solution of 0.25 *M* sucrose + 0.05 *M* Versene has a Δ value of −0.67°; the hypotonic solution of 0.18 *M* sucrose + 0.005 *M* Versene has a Δ value of −0.27°, corresponding to the lowest Δ of the substrates used in the enzymic assays.

All the enzymes were then assayed with substrates having a Δ of −0.67° for the isotonic mitochondria and supernatants and substrates having a Δ of −0.27° for the hypotonic mitochondria and supernatants. The substrates were modified in order to obtain the required cryoscopic point by adding sucrose or distilled water, as shown in the next section, in order to avoid a common mistake in the enzymic determinations. In

fact the mitochrondria isolated in 0.25 *M* or 0.88 *M* sucrose are often tested for their enzymic behavior with a reaction mixture, usually very hypotonic. Consequently, the enzymic behavior is quickly altered. The osmotic pressure of the reaction mixtures, made with chemical compounds having different dissociation constants, was determined empirically by the Beckman Cryoscope (*6*).

Enzymic Assays

The enzymic activities were constantly referred to 1 mg. of nitrogen. All the necessary blanks were run during the experiments.

Acid phosphatase

The activity of the acid phosphatase was measured according to the method of Cori and Cori (*7*). The reaction systems were incubated in an Ostwald bath for 15 minutes at 38° C. After this period 0.2 volumes of cold 50 percent trichloroacetic acid (TCA) were added, and the inorganic phosphorus was estimated in 0.5 ml. of the filtrate. The composition per ml. of the complete reaction mixture was as follows:

Hypotonic media.—1×10^{-1} *M* Na-β-glycerophosphate, *p*H 5, 0.25 ml.; 1×10^{-1} *M* acetic acid buffer, *p*H 5, 0.25 ml.; hypotonic mitochondria or supernatants, 0.25 ml.; distilled water, 0.25 ml.

Isotonic media.—The distilled water was substituted by 0.66 *M* sucrose, 0.25 ml. Isotonic mitochondria and supernatants were used.

Adenosinetriphosphatase

The ATPase activity was determined by the procedure of DuBois and Potter (*8*). To the reaction systems, after incubation in an Ostwald bath for 15 minutes at 38° C., were added 0.2 volumes of cold 50 percent TCA, and inorganic phosphorus was determined in 0.5 ml. of the filtrate. The composition for 1 ml. of the complete reaction mixture was as follows:

Hypotonic media.—1×10^{-1} *M* Na veronal buffer, *p*H 7.4, 0.30 ml.; 4×10^{-2} *M* $CaCl_2$, 0.07 ml.; 13×10^{-2} *M* Na adenosinetriphosphate, *p*H 7.4, 0.22 ml.; hypotonic mitochondria or supernatants, 0.30 ml.; 2.5×10^{-2} *M* sucrose, 0.11 ml.

Isotonic media.—The 2.5×10^{-2} *M* sucrose was substituted by 2 *M* sucrose. Isotonic mitochrondria and supernatants were used.

Cytochrome c oxidase

The cytochrome c oxidase activity was measured manometrically according to Schneider and Potter (*9*). Vessels of about 15 ml. capacity, with one side arm, were used. Temperature was 38° C.; 50 complete oscillations per minute; amplitude 3.5 cm.; KOH 2 *N* in the central well; gaseous phase = air. The side arm contained Na ascorbate. The other components of the reaction mixture were in the vessels. After 10 minutes of thermal equilibration the Na ascorbate was poured into the vessels.

Readings were taken every 10 minutes. After 30 minutes the experiment was terminated. In each experiment three different amounts of tissue were used. The autoxidation rate was calculated by extrapolating the increments in the last two vessels from the O_2 uptake in the first one (*10*). The reaction-mixture composition per ml. was as follows:

Hypotonic media.—1×10^{-1} *M* phosphate buffer, *p*H 7.4, 0.28 ml.; 2.4×10^{-4} *M* cytochrome c (Fe=0.43%), 0.28 ml.; 3.4×10^{-3} *M* AlCl 0.08 ml.; 1.14×10^{-1} *M* Na ascorbate, 0.08 ml.; hypotonic mitochondria or supernatants, 0.06, 0.11, 0.17 ml.; 0.25 *M* sucrose, 0.22, 0.17, 0.11 ml.

Isotonic media.—The 0.25 *M* sucrose was substituted by 2 *M* sucrose. Isotonic mitochondria and supernatants were used.

Analytical Methods

Nitrogen was determined by the Nessler method (*11*). Phosphorus was determined according to Fiske and Subbarow (*12*). A Unikam spectrophotometer model S.P. 400 was used.

Histologic methods.—The liver samples were fixed in Duboscq-Brasil. Sections were stained with hematoxylin and eosin. Only the results from livers showing precancerous lesions ascertained histologically were taken into account in the statistical analysis. In order to evaluate the precancerous state of the livers, we followed the criteria of Opie (*13*) and of Orr (*14*).

Electron microscopy.—The morphology of the mitochondria kept in isotonic or hypotonic media was studied by the electron microscope. A Siemens model microscope with a vibrating lens was used. A drop of a conveniently diluted mitochondrial suspension was put on a formvar-coated grid. The dried mitochondria were fixed by osmium-tetroxide vapors for 10 minutes, then washed with distilled water. The shadings were made with an alloy of gold and manganese (*15*).

Statistical analysis.—An analysis of variance according to Snedecor's method (*16*) was carried out.

Results

The results of our biochemical determinations, carried out on 40 rats (10 "Controls" on the Coward diet, 10 fed the "Deficient diet," and 10 on each diet that contained *p*-DAB whose livers showed precancerous lesions) are summaried in table 1. The nitrogen recovered in the mitochondrial fraction was, on an average, 500 to 600 μg. per ml., while that recovered in the supernatant fraction was, on an average, 80 to 100 μg. per ml. Since the presence in the supernatant of scanty, often broken, mitochondria (as seen in electron-microscopic observations) is not enough to match the nitrogen recoveries, it is most likely that most of the supernatant nitrogen belonged to the extractable portion.

Cytochrome c oxidase.—The cytochrome c oxidase activity was consistently observed in the mitochrondrial fraction; activity was never associated with the supernatant fraction, in spite of the presence

TABLE 1.—*Results of biochemical determinations on 40 rat livers (10 in each group) with precancerous liver lesions**

Enzyme activity	Controls	Coward diet + *p*-DAB	Polished-rice diet	Cantero diet (+ *p*-DAB)
Cytochrome c oxidase†				
Isotonic mitochondria	222 ± 31	218 ± 21	193 ± 23	263 ± 28
Hypotonic mitochondria	206 ± 46	344 ± 27	234 ± 29	270 ± 35
Isotonic supernatant	0	0	0	0
Hypotonic supernatant	0	0	0	0
Adenosinetriphosphatase‡				
Isotonic mitochondria	214 ± 16	321 ± 44	238 ± 23	314 ± 22
Hypotonic mitochondria	335 ± 24	370 ± 42	327 ± 24	365 ± 40
Isotonic supernatant	118 ± 23	200 ± 23	163 ± 18	196 ± 23
Hypotonic supernatant	203 ± 37	220 ± 34	184 ± 32	221 ± 29
Acid phosphatase‡				
Isotonic mitochondria	134 ± 12	93 ± 9	113 ± 6	99 ± 7
Hypotonic mitochondria	146 ± 23	99 ± 12	142 ± 13	87 ± 9
Isotonic supernatant	132 ± 18	103 ± 19	62 ± 13	55 ± 12
Hypotonic supernatant	177 ± 22	56 ± 14	71 ± 14	34 ± 9

*Each value is given as mean ± S.E.
†Expressed as μl. O_2 per hour per mg. nitrogen.
‡Expressed as μg. P per 15 minutes per mg. nitrogen.

of the previously mentioned unsedimented mitochondria. The statistical analysis shows only a highly significant ($P<0.01$) effect of *p*-DAB feeding on this enzymic activity, either associated with the deficient diet or without such association, and a threefold interaction between diet, *p*-DAB, and osmotic pressure ($0.01<P<0.05$).

Adenosinetriphosphatase.—The ATPase activity was recovered both in the mitochondrial and in the supernatant fractions. The mitochondrial enzyme was significantly affected by *p*-DAB feeding ($P<0.01$) and by the hypotonic media ($P<0.01$), while the supernatant enzyme was affected only by the action of *p*-DAB ($P<0.05$).

Acid phosphatase.—The acid-phosphatase activity was partly recovered in the mitochondrial fraction and partly in the supernatant fraction. Statistical analysis shows only a highly significant effect ($P<0.01$) of the *p*-DAB feeding on the mitochondria-bound enzyme, while the enzyme released in the supernatant is affected by many experimental factors, namely: *p*-DAB ($P<0.01$), deficient diet ($P<0.01$), and by interaction between the diet and the *p*-DAB ($0.01<P<0.05$) and between the *p*-DAB and osmotic pressure ($P<0.01$). The nature and the extent of these effects will be dealt with in the discussion, together with the results of the morphologic studies on the mitochondria.

Discussion

Adenosinetriphosphatase.—The ATPase activity is enhanced about 18 percent in precancerous liver mitochondria of rats fed *p*-DAB. This enzyme is directly affected by the carcinogenic agent without dietary interference, as confirmed by statistical analysis, which shows neither

a "diet" nor a "diet + *p*-DAB" interaction to be statistically significant. However, hypotonic media influence this activity, which is activated (table 1) to a greater extent (32%) in the liver mitochondria of rats lacking *p*-DAB in the diet than in those fed the carcinogenic agent (14%). Since there should be a mutual interdependence between the ATPase and mitochondrial morphology (*1,2*)—*i.e.*, this enzyme is activated by the swelling of the mitochondria—the lesser activation in the precancerous liver mitochondria might be interpreted as a weak response (less swelling) to hypotonic media.

Our electron-microscopic observations have in fact confirmed previous observations that, while normal liver mitochondria swell from two to three times their original volume (fig. 2) when kept in hypotonic media, the precancerous liver mitochondria show a decreased response (less swelling) to hypotonic media. This phenomenon begins to be evident in liver mitochondria of rats fed the "Coward diet + *p*-DAB" and reaches its peak in those of rats fed the "Cantero diet" (also containing *p*-DAB), which do not swell at all in hypotonic media (fig. 4). Therefore the decreased activation of ATPase in the precancerous liver mitochondria could be interpreted as an example of an enzymic alteration related secondarily to a modification of the mitochondrial structure brought about by experimental carcinogenesis.

The enzymic activity found in the supernatant is higher than expected when the low nitrogen recoveries and the small number of mitochondria present are considered. Since it is usually reported that ATPase is a structure-bound enzyme sedimented only at higher gravitational fields than those used by us (*1,2,17–20*) and that mechanical damage considerably increases its activity (*1,2*), it is likely that the marked activities found by us are due only to the presence of the fragmented mitochondria in the supernatant. The supernatant enzyme is increased by the *p*-DAB feeding in a manner (22%) not very different from the mitochondrial enzyme, but it is not modified by hypotonic media. This fact seems to support the above hypothesis that the presence of ATPase in the supernatant is due merely to the remaining mitochondria, which are predominantly fragmented and, consequently, not responsive to the variations of osmotic pressure.

Our results on the behavior of ATPase during carcinogenesis agree with those of most of the authors who found either an increased or unmodified activity in the liver of rats fed *p*-DAB (*18,20–23*) and also in mouse skin painted with methylcholanthrene (*24*). However, these results and ours are in contrast with those of Allard and Cantero (*25*), who reported decreased ATPase activity both in precancerous livers and in hepatomas induced with *p*-DAB.

Acid phosphatase.—The presence of acid phosphatase in the supernatant was expected, since this is a very diffusible enzyme, easily released by the mitochondria during the isolation procedures (*26–31*). The enzymic modifications brought about by the process of experimental carcinogenesis can be seen from the results obtained with the supernatant fraction

resulting from centrifugation following incubation in isotonic or hypotonic media, rather than from those obtained with the sedimented mitochondria with which the substrate has a limited accessibility when tested at *pH* 5 (*26–31*). The average enzymic activity for the two deficient diets ("Deficient diet" and "Cantero diet") was about 38 percent less than that for the enriched diets ("Controls" and "Coward diet+*p*-DAB"). The addition of *p*-DAB to either the enriched diet ("Coward diet+*p*-DAB") or the deficient diet ("Cantero diet") resulted in an average decrease of about 43 percent. Interaction between the diet and the *p*-DAB feeding is also suggested: Percentagewise there was a 49 percent drop in the "Coward diet+*p*-DAB" and a 34 percent drop in the "Cantero diet." In these cases, by "interaction" we mean that the carcinogenic agent acts differently when associated with different diets, strengthening the former action and slightly weakening the latter.

Osmotic pressure and *p*-DAB interact synergically, decreasing the average response about 16 percent in isotonic media and 63 percent in hypotonic media, as compared with the corresponding controls. This modified response of the precancerous rat liver is particularly interesting, since it is opposite to that of normal liver mitochondria where the release of this enzyme is usually enhanced by hypotonic media (*cf. 26–31*) or at least remains unmodified (as seen by us—*see* table 1). In fact, it could be interpreted as another consequence of the decreased mitochondrial reactivity (less swelling) to hypotonic media during carcinogenesis.

Although further studies are needed, it might be presumed that this structural rigidity is matched by decreased permeability of the limiting membrane of the mitochondria, followed by a limited release of the enzyme in the supernatant under the influence of hypotonic media. In this case too, the enzymic modification seems to be related secondarily to a precancerous alteration of the mitochondrial structure.

A 29 percent decrease of acid-phosphatase activity is caused by the effect of *p*-DAB feeding on the mitochondrial enzyme, which, although accessible to β-glycerophosphate to a limited degree at *pH* 5, obviously is influenced by the powerful action of the carcinogenic agent.

So far our results on the behavior of acid phosphatase during carcinogenesis are almost isolated; little information is given by Cantero (*32*) and by Allard (*33*), but their full paper on this work has not yet been published. According to Woodard (*34*), there is no significant difference between normal and precancerous liver activities.

Cytochrome c oxidase.—The *p*-DAB feeding increases the cytochrome c oxidase activity of the precancerous liver mitochondria about 28 percent as compared with the mitochondria isolated from livers of rats that did not receive *p*-DAB in the diet, and the results of the statistical analysis support the hypothesis of a direct action of the aminoazo-dye feeding upon this enzymic activity. The hypotonic media do not modify this enzyme despite the above-mentioned clear-cut variations of osmotic behavior of the precancerous liver mitochondria.

No enzymic activity was found in the supernatant, probably because

the remaining mitochondria were too few to develop activity which was manometrically recordable; moreover, they were mostly fragmented, and it is known that cytochrome c oxidase is affected by mechanical damage of the mitochondria (*35*).

The absolute rates of enzymic activity are lower than those found by other authors (*18,36–38*). The decline of specific activity with time (*see* isolation procedure), the modified experimental conditions, and the age of the animals (*9*) account only partially for this discrepancy; however, the differences in technique and approach invalidate any reciprocal comparison.

As for the behavior of the cytochrome c oxidase activity during carcinogenesis, the few reports available from the literature are not in agreement and usually have not been statistically evaluated. While Cantero (*32,39*) observed decreased activity in the precancerous stages, Hogeboom, Claude, and Hotchkiss (*40*) found no changes in the Q_{O_2} nor in the percent recovery of this enzyme in rats fed *p*-DAB for 50 days, as compared with controls; on the contrary, the results of Carruthers and Suntzeff (*41*), who reported increased activity in precancerous mouse skin painted with methylcholanthrene, are similar to our own.

Conclusions

These studies show that, during carcinogenesis, some enzymic activities, *e.g.*, ATPase, acid-phosphatase, and cytochrome c oxidase, are modified either in a positive or negative direction.

While *p*-dimethylaminoazobenzene (*p*-DAB) consistently affects these enzymes, the employed deficient diet is effective only in decreasing acid-phosphatase activity.

Structural modifications of the mitochondria are brought about by experimental carcinogenesis, *i.e.*, decreased response (less swelling) to hypotonic media, as compared with the controls. As a result of this structural rigidity, found following *p*-DAB feeding, ATPase and acid-phosphatase activities, which are usually enhanced by keeping the mitochondria in hypotonic media, show weak activation as compared with the controls (no *p*-DAB feeding). This fact may suggest that some aspects of the precancerous enzymic modifications are related secondarily to structural alterations caused by the carcinogenic agent.

This hypothesis cannot be generalized, however, since the cytochrome c oxidase, for instance, is not modified by morphologic variations produced by keeping normal and precancerous liver mitochondria in hypotonic media.

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PLATE

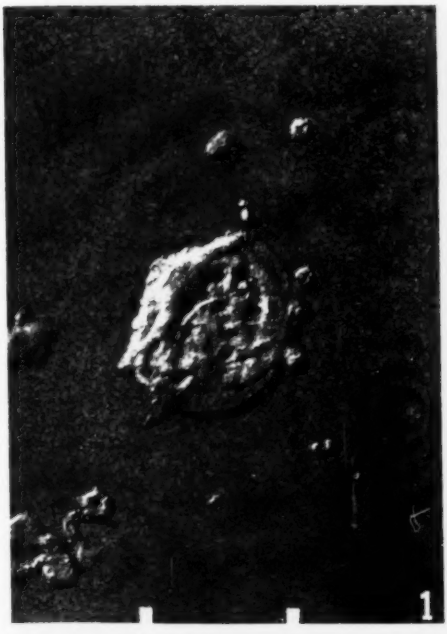
PLATE 129

FIGURE 1.—Mitochondria of normal rat liver isolated in isotonic medium. × 15,000

FIGURE 2.—Mitochondria of normal rat liver, suspended in hypotonic medium. They swell from two to three times their original volume. × 15,000

FIGURE 3.—Liver mitochondria of rats fed *p*-DAB for 4 months isolated in isotonic medium. × 15,000

FIGURE 4.—Liver mitochondria of rats fed *p*-DAB for 4 months suspended in hypotonic medium. They are not swollen as compared to those isolated in isotonic medium. × 15,000



Clerici and Cudkowiez 1471

379436—56——18

In Vitro Studies on the Iso-Antigen System in Mice [1,2]

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The genetic basis for tissue transplantation has been well established (*1,2*). These genetic laws have been most clearly defined for homotransplantation in mice by Snell and co-workers (*3–6*). The multiple dominant genes involved have been referred to as histocompatibility genes by Snell (*3*). Gorer (*7*), who studied red-blood-cell groups in mice, first put forth the hypothesis that the genes determining red-cell iso-antigens were identical with those determining resistance or susceptibility to homografts. In 1948, Gorer, Lyman, and Snell (*8*) demonstrated that the histocompatibility-2 (*H-2*) gene of the mouse did indeed determine certain red-cell antigens, and that it also showed linkage with the gene for fused tail (*Fu*), which thus placed this locus in the ninth linkage group (*9*). Tumors representing the *H-2* allele of one strain of mice, when injected into mice identified by other *H-2* alleles, will regress, and antibodies to the red cells of the tumor strain of mice will be demonstrable. Further analyses of the histocompatibility genes have been made (*10–13*), and there have also been advances in the serologic detection of the iso-antibodies (*14,15*).

Despite the appearance of isohemagglutinins in the serum of the host following regression of a tumor from a foreign strain, it has not been demonstrated that these antibodies are responsible for the destruction of the homograft. In general, the circumstantial evidence is strong for the primary role of the antibody response in the rejection of homologously transplanted tissue, but the *prima facie* evidence, the *in vitro* demonstration of cytotoxic antibodies, is lacking.

The experiments herein described were undertaken to determine whether the isohemagglutinins of mice, alone or in combination with cells from the immunized animals, are cytotoxic for tissue of the donor strain grown *in vitro*.

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Procedure

Animals.—Mice of the C57BL/10JBs strain and the isogenic subline C57BL/10–*H-2*^{d} were obtained from the Roscoe B. Jackson Memorial Laboratory through the courtesy of Dr. P. R. F. Borges and Miss G. E. Foerster. The C57BL/10JBs strain (hereafter called B10J) is characterized by an *H-2*^{b} allele in contrast to that of the subline C57BL/10–*H-2*^{d} (hereafter called B10B). A mammary carcinoma indigenous to the B10J line, called B3, was also supplied. It has been found that this tumor has a 100 percent transplantability rate in the strain of origin, while the incidence of takes in the isogenic subline is 0 percent (*16*). This tumor was received in its 111th transplant generation and was maintained by transplantation every 2 weeks into healthy B10J adults.

Immunization procedures.—One set of 40 B10B mice was immunized with normal B10J tissues (this set will be referred to as the I. N. set). Another set of 40 B10B mice was immunized with B3 tumor tissue (this set will be referred to as the I. T. set).

Antigen for the I. N. mice was prepared by removing the axillary and inguinal lymph nodes, the spleen, liver, and thymus from a normal B10J mouse and mincing these finely in a petri dish to which approximately 0.5 ml. of Gey's balanced salt solution had been added. One B10J mouse provided tissue sufficient to inject 20 recipients with 0.1 ml. each.

Antigen for the I. T. set was the B3 tumor, removed after 12 to 16 days' growth and prepared as above. The immunization schedule for both sets was 1 weekly intraperitoneal injection for 3 weeks, then 1 subcutaneous injection 1 week later. For each injection, 0.1 ml. of tissue was used. If more than a month had elapsed since the last tissue inoculation, the animals received a 0.1 ml. booster intraperitoneally before further bleeding. Bleeding was from the tail vein, and to the serum obtained 200γ per ml. of neomycin was added before storage at −22° C. in 0.25 ml. aliquots.

Tissue-culture procedure.—The B3 tumor and adult renal papilla from B10J mice were the tissues grown *in vitro* for the study of cytotoxins. The B3 tumor was soft and hemorrhagic *in vivo*. It was removed 14 to 16 days after transplantation to the B10J host. The tumor was minced and washed in Gey's balanced salt solution and then incubated for 20 minutes in an equal volume of 2 percent crude trypsin at 37° C. The tissue was washed twice again with the balanced salt solution. When the mass of tissue was centrifuged after trypsin treatment, four layers formed: the fluid supernatant, a layer of amorphous material, red cells, and the bottom layer, containing most of the tumor cells. All except this last layer were discarded. An equal volume of chick-embryo extract was then added to the remaining sediment, and 1 drop of this suspension was placed on an 11 × 22 mm. coverslip and mixed with 1 drop of chicken plasma (to which neomycin, 200γ per ml., had been added) to make a firm clot. Two coverslips, back to back, were placed in a roller tube, and, after standing 2 hours at room temperature, fluid nutrient medium was added. With this technique it was possible to obtain solid sheets

of epithelial cells. There was comparatively little growth of fibroblasts. The clot was usually lysed after about 10 days' growth while the cells continued to grow on the glass coverslip. *In vivo* growth of the tumor was established in 12 out of 12 mice with these cells grown in tissue culture.

Renal papillae were removed from the kidneys of adult B10J mice and cut into explants about 1 × 1 mm. square. One explant was placed on an 11 × 22 mm. coverslip in a clot composed of 1 drop of chick-embryo extract and 1 drop of chicken plasma (plus neomycin). The subsequent handling was as described above. After 6 to 10 days, an excellent pavement of epithelial cells was usually present. Fibroblast growth was never found.

The fluid nutrient in all cases was Gey's balanced salt solution 50 parts, human ascitic fluid 45 parts, chick-embryo extract 5 parts, plus penicillin 1,000 units per ml. In the case of the renal papillae, it was found that if the human ascitic fluid was heated to 56° C. for 3 hours, the troublesome lysis of the clot and subsequent loss of the explant before growth began could be avoided (*17*). Two ml. of the nutrient were added to each roller tube and changed twice a week. For testing procedures several different nutrients were employed, as will be mentioned below.

Testing procedures.—For testing, the coverslips were removed from the roller tubes and, to avoid an excess of antigen, the explants were removed, leaving only the outgrowth. In the case of the sheet-like growth of the carcinoma cells, all outgrowth except a central 2 × 2 mm. patch was scraped away. Each coverslip was then placed in a flat-sided roller tube, the Leighton tube. The fluid nutrient consisted of 50 parts B10B mouse serum, normal or immune, and 50 parts Tyrode's solution with 4 times the normal amount of glucose, as recommended by Parker (*18*), for a total volume of 1 ml. Phenol red was used in a concentration of 0.005 percent. Fragments of lymph node or spleen were added according to the design of the experiment.

Other techniques were used in attempting to elicit a cytotoxic effect. Spleen or lymph-node explants were placed in a clot on a coverslip and set back to back in a roller tube with a coverslip containing the test tissue. Closed-chamber techniques were also employed. The coverslip with the test tissue was placed on a 2 × 3 inch glass slide in a rectangle of wax. Serum and cells were added and all were sealed with a 24 × 60 mm. coverslip fixed to the wax outline. Observations were made by phase-contrast microscopy in most cases at 4- and 24-hour intervals. A total of 249 cultures was studied.

Agglutination tests.—The test for isohemagglutinins was essentially that of Gorer (*14*) as described by Hoecker, Counce, and Smith (*15*). A 2 percent solution of washed B10J red blood cells was prepared in human serum diluted 1 : 2 with saline. The human serum, inactivated by heating to 56° C. for 30 minutes, had been absorbed twice with an equal volume of pooled mouse red blood cells to remove agglutinins. The antiserum was diluted in a 2 percent dextran solution prepared by diluting a 10 percent solution of dextran with normal saline. The dextran used was Glaxo's

Intradex 10 percent in 5 percent glucose (salt free).[5] Other dextrans used were not satisfactory. Doubling dilutions of antiserum were prepared in the range to be studied and the mixture of antiserum and red blood cells was incubated at 37° C. for 1 hour in small precipitin tubes. Suitable controls were included. The contents of the tubes were then placed on glass slides and the results read microscopically.

Results

Agglutination titers.—The serum to be used for the study of cytotoxic action was first tested for hemagglutinin activity (table 1). It can be seen that both the I. T. and the I. N. sera gave high titers. The I. T. serum always titered as well as or better than the I. N. serum, but the difference was slight in every case. The antibody activity of the serum was preserved by storage in the deep-freeze unit, but at ordinary refrigerator temperatures it lost its potency rapidly.

The Effect of Serum from Immunized Animals on B10J Cells in Vitro

A. Effect on normal cells.—The I. N. and I. T. sera were diluted 1:2 in balanced salt solution and added to cultures of renal papillae on coverslips in Leighton tubes. The cultures were examined 4, 24, and in some cases 48 hours later by phase-contrast microscopy. No cytotoxic activity was evident. No difference was seen between the cultures treated with immune sera and those treated with normal B10B serum. The use of undiluted antiserum was similarly without effect on these cells.

B. Effect on carcinoma cells.—In the same manner, I. N. and I. T. sera were added to cultures of B3 mammary carcinoma. The use of these antisera, and normal B10B serum as well, caused the appearance of sudanophlic vacuoles in these cells within 4 hours and destruction by fatty degeneration in 24 to 36 hours. Niven (*19*), in 1929, reported a similar situation with a mouse carcinoma grown in homologous media. This effect was not due to some nonspecific factor in the serum of the resistant strain, since it occurred also with serum from the susceptible strain B10J. These vacuoles appeared when concentrations of mouse serum varying from 5 to 100 percent were used. The effect was not eliminated by heating the serum to 56° C. for as long as 2 hours. However, old serum, kept in the refrigerator at 4° C. for 3 months, did not have this effect. The B3 carcinoma grew well in nutrient prepared with protein from heterologous sources.

The Effect of Serum and Cells from Immunized Animals on B10J Cells in Vitro

A. Effect on normal cells.—Spleen and lymph nodes were removed from immunized B10B animals 4 to 6 days after the last booster injection. Normal B10B mice were used to provide tissue for the controls. Fragments of each type of tissue were minced and placed in Leighton tubes

[5] The dextran solution was provided through the courtesy of the Glaxo Laboratories, London, England.

TABLE 1.—*Agglutination of C57BL/10JBs red blood cells by antisera prepared in C57BL/10–H-2^d mice*

	Control	Agglutination of red cells by antibody diluted 1:														
		2	4	8	16	32	64	128	256	512	1028	2056	4112	8224	16448	32896
I. N. serum*.....	0	0	++	+++	+++	+++	++++	+++	++	++	+	+	±	±	0	0
I. T. serum†.....	0	0	++	++	+++	+++	+++	+++	+++	++	+	+	++	+	+	±
Normal serum‡..	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*Serum from C57BL/10–*H-2^d* mice immunized with C57BL/10JBs normal tissue.
†Serum from C57BL/10–*H-2^d* mice immunized with C57BL/10JBs carcinoma (B3).
‡Serum from untreated C57BL/10–*H-2^d* mice.

containing cultures of B10J renal papillae and nutrient media prepared with serum from immunized animals. The combinations of immune material used in these tubes were serum and spleen, serum and lymph node, and serum with explants of both spleen and lymph node. Duplicate trials were run using serum and cells from each immunized set (I. N. and I. T.). Experiments were also carried out using immune serum and immune thymus, immune serum and immune liver, and immune serum plus equal parts of immune spleen, lymph node, thymus, and liver. In order to obtain reliable and replicate results, it was very important to weigh the tissue fragments used in the experiments. When a total of 35 mg. of tissue and 1 ml. of nutrient medium was used, there was no difference between the immunized and control cultures after 24 hours. If kept longer than this time, the drop in *p*H and depletion of nutrients caused nonspecific changes in the cultures. When a total of 50 or 75 mg. of immune or normal tissue was used with 1 ml. of nutrient, all cultures were destroyed in 24 hours. When this amount of tissue was used with 2 ml. of fluid-nutrient medium, there was a moderate accumulation of fatty vacuoles in the B10J cells after 24 hours' incubation but no effect that could be attributed to specific cytotoxins.

B. Effect on mouse carcinoma cells.—Because of the deleterious effects of normal mouse serum on these cells *in vitro*, as mentioned above, it was necessary to conduct these experiments with nutrient media prepared from heterologous species. Experiments were first carried out in the stock nutrient medium, prepared with human ascitic fluid as previously described. To the cultures of B3 carcinoma in this medium were added lymph node, spleen, thymus, and liver from immunized animals, one type of tissue for each culture. In addition, combinations of lymph node and spleen and equal parts of each of the four tissues were added to similar cultures. No specific injurious effect was noted after 24 hours. It was felt that protein from sources other than human ascitic fluid might be necessary for the production of cytotoxins by tissues from the immunized animals. Human serum, horse serum, rabbit serum (inactivated), and beef amniotic fluid were tried, with and without the addition of chick-embryo extract, with no change in the results.

Effect of Extracts of Immune Tissue on B10J Cells in Vitro

Spleens and lymph nodes were pooled from 6 immunized (I. N.) mice 4 days after the last booster injection. The spleens and lymph nodes of the immunized animals were usually hypertrophic. The total weight of the spleens was 2.37 gm. and the lymph nodes, 0.51 gm. These tissues were minced and ground in a Potter tissue grinder in 3 ml. of immune (I. N.) serum. The suspension was centrifuged and a cell-free, opalescent supernatant fluid was removed. This extract was then mixed with balanced salt solution in varying concentrations: 10, 20, 40, and 60 percent. These solutions were used as the fluid media for B10J renal-papilla cultures 8 days old. There were no significant effects after 24 hours.

Production of hemagglutinins in vitro.—Since no cytotoxins were found

in the immune serum, and none were produced by the addition of immune splenic or lymphoid cells, it seemed pertinent to determine whether the experimental conditions used here were satisfactory for the production of any type of antibody *in vitro*. To this end, immune spleen and lymph-node fragments were first washed several times with Gey's solution to minimize the possibility of false positive results from small quantities of adhering immune serum. The explants were then incubated with normal B10B serum in Leighton tubes and 24 or 48 hours later the fluid was removed, centrifuged free of cells, and tested for the presence of hemagglutinins. As noted in table 2, a moderately high titer of hemagglutinin activity was found with normal serum incubated with immune lymph node or spleen, but none was found in the serum incubated with normal lymph node and spleen.

TABLE 2.—*Agglutination of C57BL/10J Bs red blood cells by normal serum* incubated with immunized C57BL/10*-H-2^d *splenic and lymphoid cells*

	Agglutination of red cells by antibody diluted 1:										
	Control	1	2	4	8	16	32	64	128	256	512
Normal serum + I. N.† spleen‡	0	0	±	+	++	++	±	0	0	0	0
Normal serum + I. N. lymph node§	0	0	0	+	+	+	++	+	0	0	0
Normal serum + I. N. lymph node and spleen‖	0	0	0	0	+	+	+	++	+++	+	+
Normal serum + normal lymph node and spleen¶	0	0	0	0	0	0	0	0	0	0	0
I. N. serum	0	0	0	++	+++	+++	+++	++	+++	+++	++

*Serum from untreated C57BL/10-*H-2^d* mice.
†I. N. refers to material from C57BL/10-*H-2^d* mice immunized with C57BL/10J Bs normal tissue.
‡Serum tested after 24 hours' incubation with 50 mg. of spleen.
§Serum tested after 24 hours' incubation with 50 mg. of lymph node.
‖Serum tested after 48 hours' incubation with 75 mg. of spleen and lymph node.
¶Serum tested after 48 hours' incubation with 75 mg. of spleen and lymph node from untreated animals.

Discussion

Medawar (*20*) reported that serum from rabbits in which skin homografts had regressed was not toxic for rabbit skin grown in tissue culture. There are other reports on the inability to form cytotoxic or neutralizing antibodies in homologous animals (*21,22*). On the other hand, Lumsden (*23*) claimed that he was able to develop homologous cytotoxins for cancer tissue. Phelps (*24*) stated that he also produced cytotoxins in homologous hosts, but without the specificity claimed by Lumsden. We endeavored to follow the conditions laid down by Lumsden for the demonstration of these antibodies as far as was practicable; *e.g.*, the explant was removed to avoid antigen excess; when a clot was present at the time of testing it

was removed; whenever possible nonhomologous material such as embryo extract was eliminated; in some cases mouse serum was used immediately after its separation without storage; experiments were conducted in closed chambers to establish the partial anaerobiosis considered important by Lumsden in order for the leukocytes to manifest cytotoxic activity. In addition, renal papillae from adult mice were used because fetal tissue, although it grows better in tissue culture, has been shown to be lacking in some antigens (*25*). None of these measures proved successful.

With regard to the use of tissue other than that used as antigen to test for the presence of cytotoxins, genetic differences certainly are well marked in epithelial cells. Homologous transplants of C57BL/10JBs skin to the isogenic subline were rejected in 12 out of 12 grafts. Isologous transplants of C57BL/10JBs skin were successful in five out of six trials. Of course, one is limited in the types of cells that may be grown *in vitro*, particularly when the tissues are taken from adult animals. However, when the tests for cytotoxins were carried out with epithelial cells and fibroblasts from the same mouse carcinoma as that used for immunization, the results were likewise negative. In other experiments it has been shown that antiserum formed against malignant cells was damaging to normal tissue as well (*24*).

The role of the lymphocyte appears primary in the homograft reaction. It is the most common cell in the inflammatory response to homografts, in contrast to the neutrophils, which predominate in the reaction to heterologous-tissue grafts (*26,27*). Weaver, Algire, and Prehn (*28*) found that when tissue was placed in the peritoneal cavity of immunized homologous animals in containers that permitted fluid but not cells to enter, the tissue survived. But the tissue was destroyed when placed in containers that permitted host cells as well as fluid to enter. Mitchison (*29*) transferred immunity to lymphosarcoma cells by transplanting lymph nodes from immunized to untreated animals. A number of other cases may be cited showing the production of antibodies following the transplantation of immunized lymph nodes or spleen (*30–33*). When taken from immunized animals, these tissues have been shown to be capable of producing antibodies *in vitro* (*18,34–38*). Kidd and Toolan (*39*) were able to "neutralize" mouse lymphosarcoma cells by incubating them for 1 hour with lymphoid cells from animals in which the sarcoma had regressed. Pomerat (*40*) found that the addition of splenic cells enhanced the action of antiserum against a rat sarcoma. However, Harris (*41*) found, "Cytotoxic phenomena are likewise absent when mixed cultures are made, in this immune mouse serum, of the mouse tumor and spleen or lymph nodes from immune mice." In addition, Harris states that he and M. R. Lewis repeated these experiments in rats with the same findings.

Our results, then, corroborate the findings of Harris and Lewis. Under genetically defined and rigidly controlled experimental conditions, mouse isohemagglutinins were found to have no cytotoxic activity, nor did the addition of splenic or lymphoid cells from immunized animals impart such activity, although these cells were shown to be capable of producing or

liberating agglutinating antibodies *in vitro*. It is possible, therefore, that the immune cells produced one type of antibody and not another. Mitchison and Dube (*42*) have recently introduced evidence indicating that the hemagglutinating antibody and the antibody responsible for homograft resistance are two separate and distinct immune substances. However, an alternate consideration is that the agglutinins or a second antibody do have cytotoxic activity, but it is not manifested under the conditions of these experiments. The antibodies with cytotoxic activity may be formed against intracellular constituents and unable to express themselves in the presence of intact cells. Chronic rather than acute contact of the test cultures with antiserum may be important in two ways. It may require prolonged and repeated application of antibodies to exert an effect. This point was investigated in a small number of experiments by replenishing antiserum in the roller tubes every other day for 10 days. No effect was noted. After this time there may be nonspecific changes in the cells of the culture. Contact over an extended period of time may also be important in that cytotoxins, particularly if they are directed against intracellular antigens, may be more effective during certain phases of cellular activity—mitosis, for example. Although these experiments offer no evidence on this point, Medawar (*26*) has reported some observations that tend to support this view. He found that the reaction of a host to a second-set graft was not manifest for at least 4 days after transplantation, at which time cellular reproduction in the graft was just commencing.

Billingham and Sparrow (*43*), with skin epithelial cells, and Mitchison and Dube (*42*), with mouse sarcoma cells, have reported that incubation of these cells with immune serum inhibits their growth on homotransplantation. However, their experiments do not allow the conclusion that a serum cytotoxin is directly responsible for the results obtained. The action of the immune serum on the cells prior to transplantation may just be part of the total host reaction. One must consider that the mechanism of destruction of homografts differs from that of heterologous transplants where the directly deleterious effects of cytotoxins have been demonstrated (*19,40*). Damage to homografts, following antigen-antibody combination, may be mediated *in vivo* through some as yet undefined factor that is absent in tissue culture.

Summary

1) High titers of isohemagglutinins were produced in C57BL/10–*H-2*^{d} mice by injection of normal and carcinoma tissue from the C57BL/10JBs line.

2) These isohemagglutinins had no cytotoxic activity, under the experimental conditions described, upon cells of the donor line grown *in vitro*.

3) The addition of splenic and lymphoid cells from the immunized animals, or extracts of these tissues, did not impart cytotoxic activity to normal or immune mouse serum.

4) Cultures of an adenocarcinoma from the C57BL/10JBs strain were

observed to undergo fatty degeneration when exposed to nutrient media prepared with normal or immune mouse serum from the strain of origin or the isogenic subline.

5) Spleen and lymph-node fragments from the immunized animals were shown to produce isohemagglutinins *in vitro*.

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Benzo[*a*]pyrene Content of Certain Pyrogenic Materials[1]

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The presence of benzo[*a*]pyrene (hereafter abbreviated as Bp) in soot (*1,2*), in tars of various kinds (*3,4*), and in other materials (*5–10*) obtained by the pyrolysis of organic materials suggests that this potent carcinogen may be present in many other products obtained from a great variety of sources by heat treatment.

The present paper discusses the quantitative analysis of Bp in a number of such products; *viz.*, Japanese domestic fuels, scorches of flour and wood soot, and combustion products of cigarette and cigar tobacco. For comparison, different types of coal soot and combustion products of gasoline have also been analyzed for Bp.

Experimental

Materials

Reagents

Benzene.—Reagent grade, shaken with several portions of sulfuric acid until the acid layer remains colorless, then redistilled.

Petroleum ether.—Reagent grade, redistilled.

Activated alumina.—About 200 mesh, Takeda Chemical Co., Osaka, Japan, reactivated by heating.

Benzo[a]pyrene.—L. Light and Co., Colnbrook, England, purified by chromatography on activated alumina using petroleum ether containing 30 percent benzene as eluent.

Instruments

Quartz prism spectrograph.—Adam Hilger, medium dispersion (distance between 3,650 and 4,030 Å=4.1 mm.). Sakura Panchro F plates,

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[4] Director: Prof. N. Saruta.

Konishi Roku Co., Tokyo, Japan, were used with this spectrograph. The plates were developed with developer D-11, Eastman Kodak Co., Rochester, N. Y., according to the instructions of the manufacturer.

Beckman spectrophotometer.—Nonrecording.

Mercury vapor lamp.—Shimazu Seisakusho Co., Kyoto, Japan, with a filter transiting light between about 3300 and 3900 Å.

Iron spark generator.—Shimazu Seisakusho Co., Kyoto, Japan.

Sources and Preparations

Coal soot.—Collected from the chimneys of two stoves for room heating.

Combustion material of gasoline.—Obtained from the tops of pistons of automobile engines.

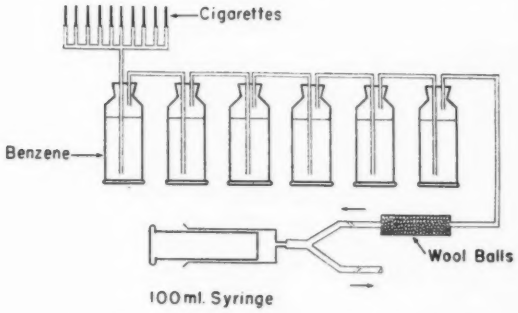
Wood soot.—Obtained from a stove used for room heating and from two water heaters in which only wood had been burned.

Charred material from biscuits and broiled sardines.—Small cakes (*ca.* 5 mm. in thickness and *ca.* 6 cm. in diameter) prepared from wheat flour containing 25 percent dextrin were heated on a wire gauze over a gas burner. The wire gauze was tightly covered with asbestos. The scorches formed were scratched off with a knife and analyzed. The sardines were heated in a similar manner and the scorches also collected.

Charcoal briquettes.—Oak charcoal briquettes of superior quality and pine charcoal briquettes of inferior quality. Both are very popular domestic fuels in Japan.

Miscellaneous fuels.—"Mametan," a molded residue of partly burned coal powder. Its production process is very similar to that of charcoal. "Gara," a sort of coke, but a little more fragile than ordinary coke. Both are popular domestic fuels in the northern district of Kyushu in Japan.

Combustion products of tobacco.—The tobacco which was used in these experiments was chosen from Japanese brands exclusively. Brands A and B are cigarette brands and C is a cigar brand. They were smoked in a smoking apparatus shown in text-figure 1 in the following manner: Cigarettes or cigars were placed in separate glass holders, lighted with a gas burner, and smoked by means of sucking air intermittently with a 100 ml. glass syringe. When the cigarettes had burned down to a length of 2 cm., they were extinguished with a drop of water. The rate of suction was about 20 times per minute and each suction was performed within a 0.9 second period on the average. During one suction nearly 9 ml. of fresh air passed through each cigarette and 20 ml. through a cigar (table 1). The generated smoke was absorbed in benzene (a total of *ca.* 900 ml. in 6 absorption flasks in series). The ashes and cigarette ends were also collected and extracted with the same solvent. The benzene extracts were evaporated. The tar obtained from the benzene solution in the absorption flasks (about 12 gm. from 400 cigarettes and about 15 gm. from 50 cigars) exhibited a strong tobacco odor. The benzene extracts of the ashes and the cigarette and cigar ends yielded only a very small amount of tar.



TEXT-FIGURE 1.—The smoking apparatus.

TABLE 1.—*Degree of ventilation during each puff by man and by the smoking apparatus*

Subject	(A) Duration of each puff (*sec.*)	(B) Volume of air aspired during each puff (*ml.*)	(C) = B/A Degree of ventilation during each puff (*ml./sec.*)
A	1.5±0.12 (n=8)	24	16.0
B	1.2±0.17 (n=8)	25	20.8
Smoking apparatus....	0.9±0.09 (n=11)	9	10.0

Analytical Procedure

The analytical procedure consisted of an extraction of the crude material with benzene, followed by a series of chromatographic fractionations on activated alumina. The Bp content of the chromatographic fractions was determined by means of their fluorescence spectra.

Extraction.—Finely ground samples of soot and fuel combustion residue (for amounts used consult tables 2 and 3) were extracted for 2 hours with boiling benzene in a flask attached to a reflux condenser. The extract was filtered through glass wool which had been freed from some fluorescent material by washing with benzene. The extraction was repeated with successive lots of the solvent until the extracts became nonfluorescent or almost nonfluorescent. The combined extracts were evaporated, under reduced pressure at *ca.* 60° C., to about 50 ml. Cigarette and cigar ends and their ashes were dried at 100° C. for about 8 hours in an electric drying apparatus and then extracted by the above procedure. The extracts of the fuels were almost colorless; all other extracts were dark brown.

Chromatography.—The condensed extract was passed through a small column of activated alumina. The column dimensions were 2.5 × 5 cm.

TABLE 2.—*Bp content of soots*

Material	Sample (*gm.*)	Bp (*mg./100 gm.*)
Coal soot	3. 8	5. 6
Coal soot	23. 5	1. 2
Wood soot	5. 0	3. 5
Wood soot	2. 5	3. 6
Wood soot	5. 0	2. 7
Wood soot	5. 0	3. 5
Wood soot	12. 0	0. 17

in most cases. Somewhat larger columns were required when the benzene extract contained considerable amounts of solute. The size of the column was adjusted so that the filtrate was yellow or light brown. A dark-brown filtrate indicated that the column used was too small. The column was washed with about 300 ml. or more of benzene until the filtrate became almost colorless and the fluorescence of the filtrate observed in ultraviolet light very weak. A large portion of the acid and alkaline substances and certain high molecular hydrocarbons were thus retained near the top of the column, while Bp was readily eluted. The filtrate was evaporated under reduced pressure at 50 to 60° C., and the oily residue was extracted with 5 to 6 successive lots of boiling petroleum ether. The last extract was colorless and only faintly fluorescent in ultraviolet light. The combined extracts were pale straw-colored and strongly blue fluorescent solutions. They were concentrated to a small volume and then rechromatographed on activated alumina. Columns measuring 1.2 × 10 cm. were used in most cases; columns measuring 1.5 × 15 cm., in the case of tobacco tar. After the petroleum-ether extract was placed on the column and before elution was started, the solute did not occupy more than about one fifth of the length of the column. The chromatogram was developed with petroleum ether, until the eluate became almost nonfluorescent. Then the development was continued with petroleum ether containing 30 percent benzene. Inspection of the column in ultraviolet light revealed the presence of a number of differently colored, fluorescent or nonfluorescent zones—moving fast, slowly, or not at all. Any Bp present is usually associated with a moderately fast-moving yellow fluorescent zone bordered on both sides by purplish-blue fluorescent zones. Sometimes, however, the yellow

TABLE 3.—*Bp content of combustion products of gasoline obtained from the automobile engines*

Car	Octane value of gasoline used	Sample (*gm.*)	Bp (*μg./100 gm.*)
A	Unknown	10	140
B	80	43. 5	9
C	76	25. 5	0
D	72	8. 2	24
E	72	2. 8	0

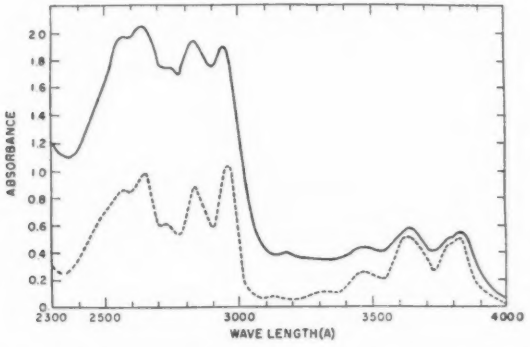
fluorescent zone was missing and only a purplish-blue fluorescent zone moving at the same speed could be observed. A large number of eluate fractions were collected. Those in which the presence of Bp could be suspected, and some adjoining fractions, were combined and slowly evaporated at about 50° C. under reduced pressure. The residue was redissolved in about 10 ml. of petroleum ether. This petroleum-ether solution was placed on a smaller and a little longer column of activated alumina (*ca.* 0.8 × 15 cm. in most cases) and in addition a third chromatography on activated alumina was also carried out in the same manner as the second.

Again a large number of eluate fractions, *ca.* 15 ml. each, were collected. The fractions in which the presence of Bp could be suspected were combined and evaporated. The two fractions collected immediately before and the two fractions collected immediately after the collection of the fractions suspected of containing Bp were evaporated separately. The residues from all three fractions were dissolved in a known amount of petroleum ether and examined fluorospectroscopically. The amount of petroleum ether used depended on the intensity of fluorescence. Fifty ml. were used for fractions from coal and wood soots containing much Bp and 3 to 5 ml. for fractions containing relatively little Bp, *e.g.*, those obtained from tobacco products. Fractions in which the detection of the fluorescence spectrum of Bp was hindered through the presence of an overlapping, broad, diffuse fluorescence band were once more chromatographed in the same manner as before but a somewhat longer column was used. An extraction with cold concentrated H_2SO_4, as suggested by Berenblum, rarely yielded satisfactory results.

Fluorospectroscopy.—A quartz test tube (inside diameter: 8 mm.) containing the petroleum-ether solution was placed at a distance of 5 mm. from the slit of a quartz spectroscope. The solution was illuminated at a 90° angle by an ultraviolet-light beam (3300–3900 Å) from a mercury arc lamp. Eight to nine fluorescence spectra were recorded on each plate, together with the fluorescence spectrum of a solution of pure Bp, and an iron spark spectrum which serves to measure exactly the wavelengths of the various fluorescence bands. A 0.075 mm. slit-width and 30-minute exposures were used. The exposure time for the iron spark was 3 seconds. Although Schoental and Scott (*11*) showed that the fluorescence spectrum of Bp consists of 7 bands, only 6 of them, *viz.*, those at 4032, 4082, 4149, 4272, 4310, and 4367 Å, appeared on the plates when Bp was used at a level of 1 μg. per ml. At lower levels all these bands gradually faded; at a level of 0.31 μg. only the 4032, 4082, and 4272 Å bands appeared distinctly; at a level of 0.16 μg. per ml. only the 4032 Å band appeared distinctly, the 4272 Å band weakly, and the 4082 Å band very weakly; and at a level of 0.08 μg. per ml. only the 4032 Å band could barely be detected.

In order to estimate the Bp content of the various solutions, the intensities of the fluorescence bands were visually compared with those of a series of standard solutions containing from 0.08 to 1.5 μg. Bp per ml.

Absorption spectrophotometry.—Whenever the identification of Bp by means of fluorospectroscopy alone was uncertain, the absorption spectrum in the ultraviolet range was examined with ethyl ether as solvent. Text-figure 2 shows the absorption spectrum of an eluate from coal soot, together with the spectrum of pure Bp. It can be seen that all absorption peaks of Bp also appear in the spectrum of the eluate.



TEXT-FIGURE 2.—The absorption spectra of an eluate from coal soot (*solid line*) and of pure Bp (*dash line*).

Results

Analysis of Combustion Products

Soot of Coal and Gasoline

In 2 samples of coal soot of different origin, 5.6 and 1.2 mg. Bp per 100 gm. of soot were found (table 2). In 3 out of 5 samples of combustion material of gasoline small amounts of Bp, ranging from 9 to 140 μg. per 100 gm. were found (table 3).

Vegetable Soots

a) *Wood soot.*—Five samples of wood soot contained from 0.17 to 3.6 mg. of Bp per 100 gm. of soot (table 2).

b) *Charred material from biscuits and from broiled sardines.*—That small amounts of Bp can be formed during a baking process is demonstrated by the fact that 7.2 and 1.1 μg. Bp were found in 100 gm. of charred matter from biscuits. On the other hand, no Bp was found in the scorches from broiled sardine.

c) *Charcoal briquettes.*—The analyzed oak charcoal briquettes contained 25 μg. of Bp per 100 gm., while Bp could not be detected in the pine charcoal briquettes (table 4).

Miscellaneous Fuels

Gara was found to contain 9 μg. Bp per 100 gm. Only traces of Bp (less than 0.1 μg./100 gm.) were present in Mametan (table 4).

TABLE 4.—*Bp content of domestic fuels*

Fuel	Sample (*gm.*)	Bp (*μg./100 gm.*)
Charcoal (oak)	145	25
Charcoal (pine)	14	0
Mametan	27	trace
Gara	22	9

Tobacco

The analysis of the smoke from 400 cigarettes of brand A revealed only a minimal trace of Bp. As only the strongest fluorescence band of 4032 Å could be detected, the presence of a trace of Bp appears doubtful. However, in a mixture of ashes and cigarette ends obtained from 220 cigarettes, 1.4 μg. of Bp (3.8 μg./100 gm. of cigarette ends and ashes) were found (table 5, expt. 1 and 2).

TABLE 5.—*Bp content of combustion products of tobacco*

Expt.	Tobacco	No. of cigarettes or cigars	Benzo[a]pyrene			
			Smoke (*μg.*)	Ash (*μg.*)	Cigarette and cigar ends	
					Leaves (*μg.*)	Paper (*μg.*)
1	Brand A*...	400	?†	—	—	—
2	Brand A*...	220	—	1.4 (3.8 μg./100 gm.)		—
3	Brand B*...	400	0	5.1 (2.8 μg./100 gm.)		—
4	Brand B*...	460	0	?†	—	?†
5	Brand B*...	400	0	?†	?†	—
6	Brand C‡...	50	0	0.4 (1.1 μg./100 gm.)	1.5 (1.6 μg./100 gm.)	—

*Cigarette tobacco.
†Only the band of 4032 Å faintly visible.
‡Cigar tobacco.

In the smoke of 400 cigarettes of brand B no Bp could be detected, while the combined ashes and ends contained 5.1 μg. Bp (2.8 μg./100 gm. of cigarette ends and ashes) (table 5, expt. 3).

In two additional experiments with two different batches of brand B cigarettes, the leaves and the paper of the cigarette ends were examined separately. Again Bp could not be detected in the smoke. In the ash, the paper, and the leaves only minimal traces of Bp were found. Only the strong fluorescence band at 4032 Å could be seen on the photographic plates (table 5, expts. 4 and 5).

The analysis of 50 cigars weighing 7.5 gm. each (therefore equivalent to 400 cigarettes of brand B weighing 0.9 gm. each) showed that the ashes

contained 0.4 μg. Bp and the cigar ends 1.5 μg. Bp was not found in the smoke.

Precision of Analytical Procedures

In order to determine the precision of the analytical procedure used, 4 identical samples of wood soot, each weighing 2.5 gm. were analyzed according to the described method, and 67, 99, 99, and 103 μg. Bp were found. The coefficient of variation was therefore 17 percent.

Discussion

Soots

Goulden and Tipler (*1*) detected Bp in domestic soot, Waller (*9*) and Cooper (*10*) found Bp in town air contaminated with soot. Furthermore, Waller (*9*) and Kotin *et al.* (*5,6*) found Bp in gasoline and diesel-engine exhaust gases. The presence of Bp in the black deposit on gasoline-engine pistons confirms the findings of these authors.

The present investigation shows that soots produced by the combustion of wood contain almost as much Bp as coal soot. In this connection the observation of Sulman and Sulman (*12*) that wood soot obtained from the chimney of a smoked-sausage factory elicited cancers, when applied to the skin of rats and mice, is of interest.

As all the soots which are produced by combustion of domestic fuels and gasoline and which are emitted freely into the air contain Bp, they might be regarded as one of the factors probably responsible for the production of human lung cancer. Furthermore, the fact that some Japanese domestic fuels contain a small amount of Bp prior to their combustion suggests that the dusts of these fuels present a cancer hazard, particularly to workers in fuel factories.

Scorches of Wheat Flour and Dextrin

The biscuits used in the present investigation were heated in such a manner that direct contact with the gas flame, a possible source of traces of Bp, was avoided. Thus, the Bp discovered in the scorches must have been produced by the heating process and not by contamination. As it is customary for Japanese people to eat such scorched foods, the presence of Bp in them is of practical interest. It may have a causative relationship to the formation of certain tumors of the gastrointestinal tract. Waterman (*13*) produced stomach tumors in animals by feeding a diet containing Bp. Rusch *et al.* (*14*) and Stewart and Lorenz (*15*) elicited tumors in animals by injecting Bp under the mucous membrane of the stomach of mice. Although in these experiments considerably higher doses of Bp than those found in the charred parts of biscuits were used, it is conceivable that the persistent administration of small amounts of Bp may also be able to induce stomach tumors. This question requires further investigation.

Tobacco

Attempts have been made by several authors to detect some carcinogenic hydrocarbon, particularly Bp, in tobacco tar. While most of these attempts remained unsuccessful, minimal traces of Bp were detected by some investigators (*7,8*).

Since, in all the experiments carried out in this study, Bp was detected in cigarette and cigar ends as well as in the ashes, while no Bp was detectable in the smoke, it appears likely that the tobacco of the cigarette and cigar ends acts as a natural filter tip that retains Bp among other ingredients of the smoke. In order to investigate this question further the following experiments were carried out:

Known amounts of Bp (0.094, 4.8, and 15.0 μg., dissolved in 0.1 ml. petroleum ether) were injected into cigarettes of brand B within 2 cm. from the burning end. Then the injected cigarettes were smoked in the machine in the usual manner and the combustion products analyzed. After injection of 15 μg. Bp per cigarette (40 cigarettes), 45 percent of the total injected amount of Bp was recovered from the smoke, 8.4 percent from the leaves of the cigarette ends, and much smaller amounts from the paper and the ash (table 6, expt. 1). In 2 experiments in which 4.8 μg. Bp per cigarette were injected into 50 cigarettes, 14 and 28 percent, respectively, were recovered from the smoke, 8 and 9 percent from the leaves and considerably smaller amounts from the paper and the ash. (table 6, expts. 2 and 3). In 2 additional experiments in which only 0.094 μg. Bp per cigarette was injected into 100 cigarettes, only 3.7 percent was recovered from the smoke, 22 and 8.5 percent, respectively, from the leaves, 4 and 5.3 percent from the paper and about 1 percent from the ash.

It can be seen that the amounts of Bp in the smoke are roughly proportional to the amounts of Bp previously injected into the cigarettes. On the other hand, the percentages of Bp adsorbed by the leaves of cigarette ends are independent of the amounts of Bp injected. The percentages of Bp found in the cigarette paper and in the ashes appear to be inversely proportional to the amount of Bp injected. However, this latter finding might be subject to a considerable experimental error on account of the very small quantities of Bp contained in the paper and the ashes.

From the analysis of the combustion products of cigarettes and cigars it can be concluded that small amounts of Bp are produced by smoking, but that only a very small part of the Bp thus formed appears in the inhaled smoke. This is apparently at least partly due to the adsorption of of Bp within the cigarette and cigar ends. It may also be partly due to an escape of Bp with the uninhaled part of the smoke or to its destruction in or near the burning zone, through chemical processes taking place.

Since Bp was detected in the combustion products of cigars as well as cigarettes, it may be concluded that the cigarette paper is not essential for production of Bp. It has been suggested in recent publications (*16,17*) that it may be mainly the cigarette paper that is responsible for the frequent occurrence of lung cancer among heavy smokers. This theory is not supported by the results of the present investigation, although more

TABLE 6.—*Distribution of Bp in various combustion products of tobacco*

Experiment	No. of cigarettes	Bp injected		Bp found								Bp lost	
		Total μg.	Per cigarette μg.	Smoke		Leaves		Papers		Ashes			
				μg.	Percent	μg.	Percent	μg.	Percent	μg.	Percent	μg.	Percent
1	40	600	15. 0	270	45	50. 4	8. 4	1. 3	0. 22	0. 30	0. 05	278	46
2	50	240	4. 8	33. 6	14	21. 6	9. 0	0. 50	0. 21	0. 80	0. 33	184	77
3	50	240	4. 8	66. 5	28	19. 3	8. 0	0. 91	0. 38	0. 72	0. 30	153	64
4	100	9. 42	0. 0942	0. 35	3. 7	2. 1	22	0. 50	5. 3	0. 11	1. 2	6. 4	67
5	100	9. 42	0. 0942	0. 35	3. 7	0. 8	8. 5	0. 4	4	0. 1	1	7. 9	83

analytical data are necessary for an accurate evaluation of this problem.

Table 1 shows that the conditions related to the operation of a smoking machine are not identical with those usually prevailing in actual smoking by humans. The amount of air drawn in during each puff is higher and the duration of each puff longer in actual smoking than in the laboratory experiments presented in this paper. On the other hand, the rate of puffing is no doubt much higher in the experimental setup. The temperature within the burning cigarette or cigar depends not only on the puffing rate but also in a large measure on the degree of ventilation. As the amount of Bp and of other polynuclear aromatic compounds formed pyrogenetically depends largely on the temperature maintained during the burning process, it is evident that the small amounts of Bp found in the laboratory experiments do not necessarily indicate that Bp must also be present in the combustion products formed during the normal smoking of cigarettes and cigars. The matter is further complicated by the fact that the manner of smoking varies greatly from individual to individual.

Summary and Conclusions

Soots of coal and wood, combustion products of gasoline obtained from gasoline engines, charred material from biscuits, charcoal briquettes, and some Japanese domestic fuels were found to contain benzo[a]pyrene (Bp). A small amount of Bp was also detected in the ends and ashes of cigarettes and cigars, while no Bp could be found in the smoke.

A large part of the Bp produced by smoking is adsorbed by the cigarette ends when passing through them.

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INDEX OF AUTHORS

Volume 16

Journal of the National Cancer Institute

Page numbers in italics indicate senior authorship

Barrett, Morris K., *1305*
Beard, Dorothy, 153, 593, 1099
Beard, J. W., 153, 163, 593, 1099
Belkin, Morris, 1353
Bellamy, Albert W., 237
Bittner, John J., *1263*
Bond, Victor P., 1
Boyd, Eldon M., *913*
Boyle, Peter J., 495
Brown, David V., *1181*, *1197*
Bryan, W. Ray, *285*, *317*, *843*, 865
Bryant, Jay C., 789
Burk, Dean, 351
Burmester, B. R., *1121*
Burstone, M. S., *1149*

Cailleau, Relda, *1011*
Calnan, Dorothy, 317
Campbell, M. Elizabeth, 557, 1405
Chai, C. K., *1335*
Clark, Eloise E., 741
Clerici, Enrico, *1459*
Cole, Leonard J., *1*
Cowdry, E. V., 205
Cram, Eloise B., 471
Cudkowicz, Gustavo, 1459

Dalton, A. J., 415
Del Vecchio, Pasco R., *985*
Dennis, Don, 129, 709, 1207
Deringer, Margaret K., *763*, 1305, 1309
Dowdy, Andrew H., 237
duBuy, H. G., 173
Dunn, Thelma B., 495, 1309
Dyer, Helen M., *11*

Earle, Wilton R., 337, 789
Eckert, Edward A., *153*, *593*, *1099*
Eden, M., *541*
Ehrmann, Robert L., *1375*
Evans, Virginia J., 789
Eyestone, W. H., 723

Felix, Marie D., *415*
Fioramonti, Mary C., 789
Firminger, Harlan I., 927

Gallily, Ruth, 803, 1083
Gey, George O., 1375
Goldin, Abraham, *129*, *709*, *1207*
Gormsen, H., 1137
Green, Irving, 153, *163*, 593
Greene, Robert T., 569
Greenhouse, Samuel W., 129
Groupé, Vincent, *865*
Guerin, Laurence F., 1405

Habermeyer, John G., 1
Haenszel, William, *1417*
Haines, B., 541
Hall, George C., *237*
Hansen, Walter H., 1305
Hansen-Melander, Eva, *1067*
Hare, William V., *889*
Henderson, James H. M., 981
Heston, W. E., 763, *1309*
Hilberg, Albert W., 645, *951*, 985
Hokama, Yoshitsugi, 225
Hsu, T. C., *691*, 1047
Hueper, W. C., *55*, *447*
Humm, Douglas G., *741*
Humphreys, Stewart R., 129, 709, 1207
Hunter, Jehu C., 351, *405*

Iida, Tadao, 971

Kahler, H., 541
Kammer, Adolph G., 989
Kaplan, Henry S., 139
Kasten, Frederick H., *579*
Kirchoff, Helen, *1287*
Klein, Michael, *255*
Kline, Ira, 1353
Kondo, Tatsuhei, *971*
Kullander, Stig, 1067

379436—56——20

Kuratsune, Masanori, *1485*

Law, L. W., *495*
Leighton, Joseph, *1353*
Leiter, J., 31, 99, 1443
Levine, Alvin S., 865
Liang, Hsu-mu, *205*

McCoy, Thomas A., *1221*
McEwen, H. Douglas, 913
McQuilken, William T., 789
Main, Joan M., 1257
Mantel, Nathan, 129, 709, 1207
Melander, Yngve, 1067
Miller, Daniel G., *1473*
Milmore, Benno K., *267*
Moloney, John B., 317, *877*
Moorhead, Paul S., *1047*
Morbeck, Floyd E., 475
Morgan, Joseph F., *557*, 995, *1405*
Morris, Harold P., *489*, *927*
Morton, Helen J., 557, 995, 1405
Moss, Shirley, 1011
Mühlbock, O., 659
Mulay, A. S., *723*, 1163
Murdoch, Monica E., 913
Mustacchi, Piero O., 471

Nagareda, C. Susan, *139*
Neuman, Robert E., 1221

Ober, William B., *569*

Paradis, Anita D., 31, 99
Pasieka, Arthur E., *995*
Penn, Harry S., *225*, 237
Peppers, Ellison V., 337, 789
Pierro, Louis, 475
Poel, William E., *989*
Polissar, Milton J., 75
Powers, O., 1443
Prehn, Richmond T., *1257*
Preston, Frederick W., 1021

Quevedo, Walter C., 475

Rask-Nielsen, Ragna, *1129*, *1137*
Rauscher, Frank J., 865
Rier, John P., Jr., *981*
Rigdon, R. H., 1287
Russell, E. S., 1335

Sachs, Leo, *803*, *1083*
Saffiotti, Umberto, *961*
Sakakibara, Hideo, 971
Sanford, Katherine K., *789*
Saunders, John W., Jr., *475*
Schrek, Robert, *1021*
Shacter, Bernard, *1453*
Sharp, D. G., 153, 593
Shelton, Emma, *107*
Shimkin, Michael B., *75*, *471*, 1417
Showacre, Jane L., *173*
Shubik, Philippe, 961
Siegel, Benjamin V., 1011
Siegler, Edward E., 985
Smith, Robert R., *645*
Stewart, Harold L., 889
Stewart, Sarah E., *41*
Symeonidis, A., *1163*

Tetenbaum, Zelda, 1353
Taylor, Richard J., 569
Thorson, Theodore A., 1181, 1197

Van Rijssel, Th. G., *659*
Velardo, Joseph T., 569
Velat, Clarence A., 489
Venditti, John M., 129, 709, 1207

Wagner, Billie P., 489
Waravdekar, V. S., *31*, *99*, *1443*
Westfall, Benton B., *337*, 789
White, Philip R., *769*
Wolff, George L., *1231*, *1249*
Wollman, Seymour H., *195*
Woods, Mark, *351*
Wright, Willard H., 471

INDEX OF SUBJECTS

Volume 16

Journal of the National Cancer Institute

2-Acetylaminofluorene:
  comparison of patterns of excretion and carcinogenicity in rat, 11
  tumors induced in rats by, 489
Acetylpodophyllotoxin - ω - pyridinium chloride:
  effect of on enzymes in tumors, 99
  effect of on sarcoma 37 enzyme systems, 1443
Adrenal glands of rats, histopathologic changes produced in by diet, 1163
Adrenocortical adenocarcinoma in rats fed carcinogenic diet, 723
Amethopterin, synergistic action with 6-mercaptopurine in mouse leukemia, 129
Amino acids:
  effects of on pigment synthesis in skin, 475
  metabolism of in synthetic media, 995
o-Aminoazotoluene plus stilbestrol in carcinogenesis in mice, 107
Aminopeptidase in human neoplasms, histochemical demonstration of, 1149
Aminopterin in treatment of leukemia, 709
Announcements:
  Cancer Fellowships and Residencies, II (Dec.)
  First International Human Genetics Congress, 1307
  Gastrointestinal Cancer Conference, 591
  Grants in Cancer Research, 349, 592
  Medical Research Fellowships, 591
  Publication of Cancer Chemotherapy Screening Data, 1507
  Rhesus Monkeys for Medical Research, 591
  Third National Cancer Conference, 1307
Antigens:
  iso- ———, *in vitro* studies in mice on, 1473
  of avian myeloblastosis virus, 593
Antileukemic agents:
  amethopterin, 129
  aminopterin, 709
  6-mercaptopurine, 129
Ascites tumors:
  human ovarian cystocarcinoma, study of chromosomes in cells of, 1067
  transplantation of, into immunized mice, 1083
  see also Ehrlich ascites tumor; Krebs-2 carcinoma
Avian erythromyeloblastic leukosis: *see* Virus
Avian erythroblastosis: *see* Virus

Benzo[*a*]pyrene (3,4-benzpyrene):
  in pyrogenic materials, 1485
  in skin carcinogenesis in mice, 989
Benzoquinone, influence of on Ehrlich ascites-tumor cells, 1453
Bile-acid derivatives:
  preparation of, 225
  clinical evaluation of, 237
  role in seroflocculation reaction, 225
Bone marrow, effect of on X-irradiated mice, 1
Brain, chick, as host-virus system for Rous sarcoma virus, 865

Cancer cells, seeding of operative wounds with, 645
Carcinogenesis: *see* Tumorigenesis
Carcinogens: *see* 2-Acetylaminofluorene; *o*-Aminoazotoluene; Benzo[*a*]pyrene (3,4-benzpyrene); Chromium; 2-Diacetylaminofluorene; 1,2,5,6-Dibenzanthracene; *p*-Dimethylaminoazobenzene; 9,10-Dimethyl-1,2-benzanthracene; Methylcholanthrene; Nickel; Trypan blue
Cell nutrition:
  comparison of various synthetic media, 1405

379436—56——21

Cell nutrition—Continued
effect of serum fractions on cell proliferation, 789
growth of Walker carcinosarcoma 256 cells in fluid media, 1221
synthetic nutrients, chemically defined, 769
use of supplemented synthetic media, 557
Chemotherapy of leukemia in mice, 129, 709
Chromium and chromite ore in metal cancerigenesis of rats, rabbits, and dogs, 447
Chromosome studies:
characteristics of mitosis in HeLa cells, 1047
correlation of chromosome duplication and tumor transplantability, 803
in human ascites tumors, 10[illegible]
observations on mitosis with phase cinematography, 691
transplantability of ascites tumors into immunized mice, 1083
Compounds: *see* names of
Croton oil:
as a carcinogen in mice, 961
effects of on epithelial proliferation in mice, 255

Desmoid tumor of popliteal space in pregnancy, 569
2-Diacetylaminofluorene, induction of hepatomas with, 927
1,2,5,6-Dibenzanthracene, pulmonary tumors in mice induced with, 763
Diet:
carcinogenic, adrenocortical adenocarcinomas in rats fed, 723
low-roughage, influence of on incidence of lesions in mice, 889
semisynthetic, effect of on adrenal glands of rats, 1163
with riboflavine in tumor induction in rats receiving carcinogen, 489
p-Dimethylaminoazobenzene:
development of adrenocortical adenocarcinoma in rats fed, 723
effect on mitochondrial enzymes, 1459
histopathology of adrenal glands in rats fed, 1163
9,10-Dimethyl-1,2-benzanthracene:
compared with carcinogenicity of croton oil in mice, 961
development of leukemia in mice following injection of into thymus, 1129
9,10-Dimethyl-1,2-benzanthracene—Con.
induction of plasma-cell leukemias with, 1137
Dose-response data in experimental carcinogenesis, analysis of, 195

Ehrlich ascites tumors, influence of stilbestrol and quinone on cells of, 1453
Enzymes:
changes of in tumors with chemical agents, 31, 99
changes induced with chemical agents, 1443
mitochondrial, effects of carcinogen and other factors on, 1459

Fish, metabolism of melanomas in, 741
Fluorene compounds, comparison of patterns of excretion and carcinogenicity in rat, 11

Gastritis, chronic, pathology of in mice, 889
Genetic studies:
development of pulmonary tumors in mice in relation to three dominant genes, 763
modifiers of mouse kidney glycolysis, 1249
relationship between genotype and mammary tumor agent, 1309
relationship between obese genotype and Harding-Passey melanoma in mice, 1231
variability of mammary tumor incidence in DBA mice, 1335
Glucose utilization:
by sarcoma 37, effect of α-peltatin on, 31
in tumors, regulation of by hormones, 351

Harding-Passey melanoma:
effect of on anaerobic glycolysis in mouse kidneys, 1249
glucose utilization in, 351
relationship to obese genotype in mice, 1231
Hepatomas:
——— 129, effect of acetylpodophyllotoxin-ω-pyridinium chloride on enzyme systems of, 99
induced by *o*-aminoazotoluene plus stilbestrol in mice, 107
in rats, influence of sex and sex hormones on, 927
Histochemistry, demonstration of proteolytic activity in human neoplasms, 1149

Homotransplantation: *see* Transplantation
Hormones:
effects on utilization of glucose, 351
sex, role of in induction of hepatomas in rats by 2-diacetylaminofluorene, 927

Immunity studies:
in mice, use of diffusion chambers for, 1257
in vitro, on iso-antigen systems in mice, 1473
in vitro reaction of lymphosarcoma cells to homologous immune serum, 1021
Instrumentation:
chromatography, paper, for separation and resolution of amino acids in synthetic media, 995
cinematography, phase-contrast, in immunity studies, 1021
observations on mitosis with, 691, 1047
circling device for marking areas on slides, 985
diffusion chambers, in immunity studies in mice, 1257
electrodes, glass, for measurement of tumor *p*H, 541
microscopy, phase-contrast, in study of cells in peritoneal fluid, 415
syringe for applications of carcinogen on skin, 989
Intramandibular tumors in mice, hairs in mandible accompanying, 659

Janus green B, enzymic nature of staining with, 173

α-Keto acids in tissue culture, 337
Kidney, transplant of bone tumor to, 951
Krebs-2 carcinoma:
effect of temperature on growth in mice, 405
glucose utilization in, 351
Krebs-cycle dehydrogenases, detection of with Janus green B, 173

Leukemia:
chemotherapy of in mice, 129
development of in mice following injection of carcinogen into thymus, 1129
factors influencing chemotherapy, 709
in mice, produced by cell-free filtrates, 41
plasma-cell, in mice, 1137
——— -producing agents in mice, 41
see also Virus, Avian
Leukemic tissue, neoplasms in mice following introduction of, 495
Lipides:
effects of metabolic inhibitors on, in tissue culture, 1011
in spleen of tumor-bearing rats, 913
Lung cancer:
discussion of statistics on mortality in U. S., 267
epidemiology of and smoking patterns, 1417
Lymphomas, effect of hypophysectomy and X irradiation on, in mice, 139
Lymphomatosis, visceral: *see* Virus

Mammary tumors:
in C3H mice and hybrids, 1263
relationship of agent to genotype in mice, 1309
variability of incidence in DBA mice, 1335
Mandibular tumors: *see* Intramandibular
Melanomas:
metabolism of in fish, 741
human, glucose utilization in, 351
S91,
effects of temperature on growth in mice, 405
glucose utilization in, 351
S91A amelanotic,
effects of temperature on growth in mice, 405
glucose utilization in, 351
6-Mercaptopurine, synergistic action with amethopterin in mouse leukemia, 129
Metals: *see* Chromium, Nickel
Methylcholanthrene:
effects of on epithelial proliferation in mice, 255
role of hair follicles in carcinogenesis in mice produced by, 205
Mitochondria, characterization by Janus green B, 173
Mitosis:
characteristics of in HeLa cells, 1047
observations on with phase-contrast cinematography, 691
Mortality statistics in U. S., data available on lung cancer, 267

Nickel, powdered, cancer produced by in rats, rabbits, and dogs, 55
Nuclear size in tissues of tumorous and nontumorous mice, 579

Olive oil, effects of on epithelial proliferation in mice, 255

Parotid-gland tumors, development in mice after introduction of leukemic tissue, 495
α-Peltatin, effect of on glucose utilization and enzymes in tumors, 31
Pericarditis, calcareous, pathology of in mice, 889
*p*H of rat tumors measured *in vivo*, 541
Polyps, duodenal, pathology of in mice, 889
Promoting agents, carcinogenic action of in mice, 961
Pulmonary tumors:
development of in mice in relation to three dominant genes, 763
induction and growth of in mice with urethan, 75
Pyrogens, benzo[*a*]pyrene content of, 1485
Pyruvic acids in tissue culture, 337

Radiation: *see* X irradiation
Reticulum-cell sarcoma:
inhibition of production with trypan blue in rats by preirradiation, 1197
production of with trypan blue in rats, 1181
Riboflavine:
dietary levels and tumor induction in rats receiving carcinogen, 489
in mouse diet, test for protective action of, 107
in semisynthetic diet, effect of on adrenal glands of rats, 1163
Rous sarcoma: *see* Virus

Saliva, metabolism of thiamin in, 971
Sarcoma:
——— 37, effect of acetylpodophyllotoxin-ω-pyridinium chloride on enzyme systems of, 99
effect of α-peltatin on enzyme systems of, 31
enzyme changes in, produced with chemical agents, 1443
growth studies in mice, 1207
transplanted osteogenic, morphology of in mice, 951
Schistosoma, lack of carcinogenicity of in mice, 471
Seeding of operative wounds with cancer cells, 645
Serofloccculation reaction:
clinical evaluation of in patients with cancer, 237
Serofloccculation reaction—Continued
role of bile-acid derivatives in, 225
Skin:
carcinogenesis, quantitative approach to dose-response relations in mice, 989
effects of amino acids on pigment synthesis in, 475
Smoking:
habits of 21,612 Texans, 1287
patterns of and epidemiology of lung cancer, 1417
Spleen lipides and water in rats bearing tumor, 913
Stains: *see* names of
Stilbestrol:
carcinogenic effects of in combination with azo dyes in mice, 107
influence of on Ehrlich ascites-tumor cells, 1453
Stress, effects of on glucose utilization in tumors, 351

Temperature, effects of on growth of tumors in mice, 405
Thymus, development of leukemia in mice after injection of carcinogen in, 1129
Thiamin, metabolism of in saliva and salivary glands, 971
Tissue culture:
comparison of various synthetic media, 1405
defined nutrients for strain L cells, 769
effects of amino acids on synthesis of pigment in skin, 475
effects of metabolic inhibitors on strain L cells, 1011
effects of serum fractions on cell proliferation, 789
growth of Walker carcinosarcoma 256 cells in fluid media, 1221
α-keto and pyruvic acids in, 337
metabolism of amino acids in synthetic media, 995
plant, effect of antibiotics on bacterial contamination, 981
sponge-matrix, influence of various factors on invasive properties of HeLa, 1353
supplementation of medium M 150, 557
use of reconstituted rat-tail collagen for cell growth, 1375
Transplantation:
of ascites tumors into immunized mice, 1083

Transplantation—Continued
of osteogenic sarcoma to kidney, 951
of solid tumors related to chromosome duplication in mice, 803
tolerance of host to foreign tumor implant, 1305
Trypan blue:
production of reticulum-cell sarcoma in preirradiated rats with, 1197
production of reticulum-cell sarcoma in rats with, 1181
Tumorigenesis:
experimental, dose-response data in, 195
lack of in mice with *Schistosoma*, 471
role of hair follicles in mice in, 205
see also names and types of tumors

Urethan, induction and growth of pulmonary tumors in mice with, 75

Virus:
avian erythroblastosis,
titration of infectivity, 1099
avian erythromyeloblastic leukosis,
antigenicity and immunology, 593
dephosphorylation of inosinetriphosphate, 163
Virus—Continued
avian erythromyeloblastic leukosis—Continued
thermal stability of, 153
Rous sarcoma,
host-virus system for studies on, 865
interpretation of tumor-response data, 843
preparation of improved standard lots of, 877
recovery of from experimental tumors in relation to iniating dose, 317
review of sources of experimental variation and methods for control, 285
visceral lymphomatosis, bioassay of, 1121

Walker carcinosarcoma, 256:
analysis of spleen in rats bearing, 913
growth of cells of in tissue culture, 1221

X irradiation:
effects of in mice following administration of bone marrow, 1
effects of on mouse lymphoid organs following hypophysectomy, 139
inhibition of production of sarcomas with trypan blue in rats by, 1197

ERRATA

Journal of the National Cancer Institute, Volume 16, 1955–56

Page 49 (August 1955 issue): In the paper entitled "Neoplasms in Mice Inoculated with Cell-Free Extracts or Filtrates of Leukemic Mouse Tissues. II. Leukemia in Hybrid Mice Produced by Cell-Free Filtrates," by Stewart, in the fourth paragraph *for* "were observed in two of the inoculated" *read* "were observed in three of the inoculated."

Page 794 (December 1955 issue): In the paper entitled "The Effect of Serum Fractions on the Proliferation of Strain L Mouse Cells *in Vitro*," by Sanford *et al.*, in the footnote of table 2 *for* "indicated that 320 mg. per ml. was removed" *read* "indicated that 320 mg. per 100 ml. was removed."

Page 1149 (April 1956 issue): In the paper entitled "Histochemical Demonstration of Proteolytic Activity in Human Neoplasms," by Burstone, in the third paragraph *for* "10 ml. of 2 *M* phosphate buffer" *read* "10 ml. of 0.2 *M* phosphate buffer."

Announcement

Publication of Cancer Chemotherapy Screening Data

An Editorial Committee is now preparing another Supplement to *Cancer Research* devoted to screening data, similar to Supplements 1 and 2 published previously. Investigators are invited to submit data. The Editorial Committee calls attention to the fact that data are solicited on all biological and biochemical systems which are designed to uncover agents useful in cancer chemotherapy. These include effects on animal tumors, tissue culture, microbiological systems, biochemical systems, growth of tumors in heterologous hosts, etc. Data on clinical experience are also solicited.

The next issue will include a cumulative empirical formula index, including entries from Supplements 1 and 2. It is hoped that the inclusion of a cumulative index in the next and succeeding issues will make the specific information in these supplements more readily accessible.

The same format as in the previous supplements will be used in the forthcoming issue. It is strongly suggested that the actual evaluation measures employed in the procedure, *viz.*, the ratio of tumor sizes or survival times (treated/control) be included whenever feasible even though the materials tested are considered to be negative. Investigators are requested to follow the instructions outlined in the previous announcement (Cancer Research 14: 469, 1954).

Since one of the objectives for publishing these supplements is to provide a rapid exchange of information in this area and to provide a broader basis for eliciting structure-activity relationships, the Committee invites data on chemical agents of varying degrees of activity as well as on strictly negative agents. This should not prejudice the publication of such data in more detailed form in regular issues of other scientific publications. The Committee also solicits data on combination therapy where multiple agents are employed.

Data for publication in the next issue will be accepted until October 1, 1956. The Committee reserves the right to omit any data which provide inadequate information. The material may be submitted directly to the Editor of the forthcoming issue, Joseph Leiter, Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda 14, Maryland, or to any one of the Associate Editors listed below.

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 16 · NO. 6 · JUNE 1956

Page

Further studies on the relationship between the genotype and the mammary tumor agent in mice. *W. E. Heston, Margaret K. Deringer,* and *Thelma B. Dunn* 1309

Analysis of variability of mammary tumor incidence in inbred DBA mice. *C. K. Chai* and *E. S. Russell* 1335

Studies on human cancer using sponge-matrix tissue culture. III. The invasive properties of a carcinoma (strain HeLa) as influenced by temperature variations, by conditioned media, and in contact with rapidly growing chick embryonic tissue. *Joseph Leighton, Ira Kline, Morris Belkin,* and *Zelda Tetenbaum* 1353

The growth of cells on a transparent gel of reconstituted rat-tail collagen. *Robert L. Ehrmann* and *George O. Gey* 1375

The nutrition of animal tissues cultivated *in vitro.* **II. A comparison of various synthetic media.** *Joseph F. Morgan, Helen J. Morton, M. Elizabeth Campbell,* and *Laurence F. Guerin* 1405

Smoking patterns and epidemiology of lung cancer in the United States: Are they compatible? *William Haenszel* and *Michael B. Shimkin* 1417

Enzyme changes induced in normal and malignant tissues with chemical agents. VI. Effect of acetylpodophyllotoxin-ω-pyridinium chloride on malic-oxidase and on isocitric-oxidase systems of sarcoma 37. *V. S. Waravdekar, O. Powers,* and *J. Leiter* 1443

Interrelations in respiratory, phosphorylative, and mitotic activity of Ehrlich ascites-tumor cells: Influence of stilbestrol and quinone. *Bernard Shacter* 1453

Certain effects of *p*-dimethylaminoazobenzene, deficient diet, and hypotonic media on mitochondrial enzymes in experimental rat-liver carcinogenesis. *Enrico Clerici* and *Gustavo Cudkowicz* 1459

***In vitro* studies on the iso-antigen system in mice.** *Daniel G. Miller* 1473

Benzo[*a*]pyrene content of certain pyrogenic materials. *Masanori Kuratsune* 1485

Acknowledgment 1497

Index of authors 1499

Index of subjects 1501

Errata 1506

Announcement:
Publication of cancer chemotherapy screening data 1507